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14. ABSTRACT: The purpose of this proposal is to investigate the mechanisms involving pharmacological and behavioral enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamatedopamine interactions. This proposal has two components. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post-synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses.

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MPTP, neuroplasticity, models of injury, repair, basal ganglia

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TABLE OF CONTENTS

Introduction

The primary focus of this research proposal is to determine the underlying mechanisms responsible for neuroplasticity in the injured adult basal ganglia. For these studies we utilize the neurotoxicant MPTP that selectively destroys nigrostriatal dopaminergic neurons and leads to the depletion of striatal dopamine as well as the development of parkinsonian features. In the squirrel monkey these features include slowness of movement, balance impairment and diminished hand dexterity. In our laboratory we utilize both the MPTP-lesioned C57BL6 mouse and the MPTP-lesioned squirrel monkey. Both models show intrinsic plasticity through either striatal dopamine return (mouse) and/or behavioral recovery (squirrel monkey).   In this proposal we were particularly interested in understanding whether exercise (mouse) or dopamine replacement therapy (monkey) might enhance intrinsic neuroplasticity of the injured basal ganglia. For this purpose, the proposal was divided into two components, a mouse exercise study and a squirrel monkey dopamine replacement study. These studies were designed to be complementary in that both nonpharmacological and pharmacological effects of neuroplasticity are being investigated.

In the following sections are included the abstract, introduction and specific aims from the original proposal. This is followed by the accomplishments and research outcomes from year one. This annual report also includes manuscripts in the form of appendices.

Abstract (From the Original Application)

The purpose of this proposal is to investigate the molecular mechanisms involving pharmacological and behavioral (exercise) enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions. The following proposal has two complementary components using two animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we hope to identify novel therapeutic targets for the treatment of brain injury and neurotoxic insult. Since military personnel are at risk for a wide range of brain injuries including head trauma, neurotoxic exposure (from pesticides, hostile enemy poisoning, viral and biological weapon based agents) it is imperative that medical strategies be made available to reverse the debilitating neurological deficits.

D: STATEMENT OF WORK
From the original Application

The brain's capacity for recovery from damage is far greater than previously recognized. It is now understood that neuroplasticity can be modulated through activity-dependent processes including exercise and environmental enrichment, and through pharmacological manipulation. Most of our understanding of exercise and pharmacological enhanced neuroplasticity is derived from studies in the cortex and the hippocampus, but there is mounting evidence that the same phenomenon occurs in the injured basal ganglia. The molecular mechanisms for this phenomenon are not well understood. Using two animal models of injury induced neuroplasticity in the basal ganglia (the MPTP-lesioned mouse and MPTP-lesioned non-human primate) we propose to examine two modes of intervention to enhance neuroplasticity. These include exercise in the MPTP-lesioned mouse model and pharmacological intervention in the MPTP-lesioned non-human primate. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions.

The following proposal has two complementary components using both animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we will identify new therapeutic targets for the treatment of traumatic brain injury and neurotoxic insult, two high-risk morbidities that are common to military personnel.

Component One: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.

Component One will utilize the following 4 treatment groups for **Study1** through **Study 4**:
- (1) Saline-injected;
- (2) MPTP-injected;
- (3) Saline-injected + exercise;
- (4) MPTP-injected + exercise.

Study 5 will utilize the following glutamate antagonists: AMPA antagonist (GYKI-52466) and the NMDA antagonist (MK-801) in the following 8 treatment groups:

(1) Saline-injected + GYKI-52466;	(5) Saline-injected + MK801;
(2) MPTP-injected + GYKI-52466;	(6) MPTP-injected + MK801;

| (3) Saline-injected + exercise + GYKI-52466; | (7) Saline-injected + exercise + MK801; |
| (4) MPTP-injected + exercise + GYKI-52466; | (8) MPTP-injected + exercise +MK801. |

Exercise will be performed on a motorized rodent treadmill. Brain tissue will be collected after 30 days of running.

Study 1: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.

Study 2: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine-3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, *in situ* hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice.

Study 3: The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy.

Study 4: The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and *in situ* hybridization histochemistry.

Study 5: *We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist*. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning.

Component Two: To test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-lesioned non-human primate through its effect on dopamine (biosynthesis, uptake, and receptor expression) and glutamatergic synapses.

Component Two will utilize the following treatment groups (n = 4 per group):
(1) Saline-injected harvested at 6 weeks after the last injection;
(2) Saline-injected harvested at 16 weeks after the last injection;
(3) MPTP-injected harvested at 6 weeks after the last injection;
(4) MPTP-injected harvested at 16 weeks after the last injection;
(5) Saline-injected + pramipexole harvested at 6 weeks after the last injection;
(6) Saline-injected + pramipexole harvested at 16 weeks after the last injection;
(7) MPTP-injected + pramipexole harvested at 6 weeks after the last injection;
(8) MPTP-injected + pramipexole harvested at 16 weeks after the last injection.

Study 1: The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.

Study 2: The pattern of expression of proteins and mRNA transcripts important for dopaminergic function, (including TH, DAT, VMAT2) at the level of the SNpc and CPu will be determined. Preliminary data supports our ability to carry out western immunoblotting, immunocytochemistry and *in situ* hybridization in the MPTP-lesioned non-human primate.

Study 3: The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using *in situ* hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate.

Study 4: The effect of pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.

At the conclusion of these studies we will have a better understanding on the role of exercise and dopamine agonist (pramipexole) treatment in enhancing neuroplasticity of the injured basal ganglia in the mouse and the non-human primate. This may then identify important therapeutic targets (through glutamate and dopamine) for the treatment of brain injury.

Table 1: Timeline of Experimental Design for Component One (Exercise in the MPTP-Lesioned Mouse Model).

Component One: Exercise in the MPTP-lesioned Mouse				
	Year 1	**Year 2**	**Year 3**	**Year 4**
Study 1: Analysis of Dopamine and its metabolites	HPLC			
Study 2: Analysis of TH, DAT, CREB, and DARPP-32		Immunocytochemistry, In Situ Hybridization, Western Immunoblotting		
Study 3: Analysis of striatal glutamate synapses		Immuno-electron microscopy		
Study 4: Analysis of NMDA and AMPA receptor subtypes		Immunocytochemistry, In Situ Hybridization, Western Immunoblotting		
Study 5: Attenuate neuroplasticity with NMDA and AMPA receptor antagonists			Immunocytochemistry, In Situ Hybridization, Western Immunoblotting, Immuno-electron microscopy	

Component 2: Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.

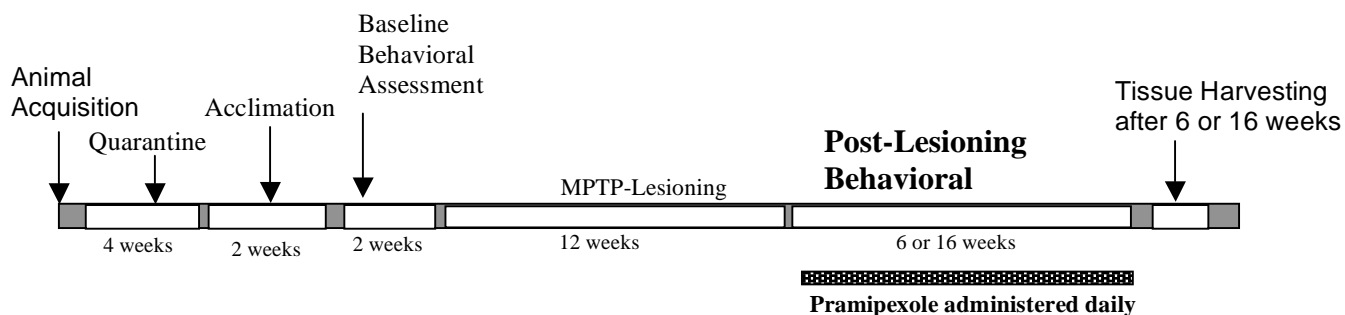### Time Line:

Table 2:

Specific Aim	Year 1	Year 2	Year 3	Year 4
	Lesion animals and administer Pramipexole			
Study 1	Behavioral analysis			
Study 2		TH, DAT, VMAT mRNA and protein using WIB, ICC and ISH		
Study 3		Dopamine Receptor D1, D2, and D3 using WIB, ICC, and ISH		
Study 4		Analysis of glutamatergic synapses using immuno-EM		

Key Research Accomplishments for Year One and Year Two

Component One: Enhancement of neuroplasticity in the MPTP-lesioned mouse

 (i) Intensive treadmill exercise improves motor performance of both MPTP-lesioned and saline treated mice. Specifically exercised animals run faster and for a longer duration. In addition, new behavioral data demonstrates that exercised animals also perform better on the accelerated rotarod, compared to non-exercised animal. This benefit is seen in both MPTP and saline treated mice.

(ii) Intensive treadmill exercise suppresses the intrinsic return of striatal dopamine transporter protein. On further analysis tyrosine hydroxylase protein does not appear to be significantly altered by exercise in the MPTP-lesioned mouse.

(iii) Intensive treadmill exercise suppresses the expression of dopamine transporter mRNA transcripts in both saline + exercise and MPTP + exercise mice. On further analysis there does not appear to be a significant reduction for the tyrosine hydroxylase mRNA transcript after exercise.

(iv) Intensive treadmill exercise causes a normalization of synaptic glutamate to levels seen in non-lesioned mice without exercise.

(v) The administration of AMPA and NMDA receptor antagonists altered the pattern of expression of tyrosine hydroxylase and dopamine transporter mRNA transcription in nigrostriatal dopaminergic neurons as well as the pattern of expression of striatal tyrosine hydroxylase.

(vi) Electrophysiological analysis of dopamine release using fast-cyclic voltammetry indicates increased release of dopamine in the MPTP-lesioned mouse undergoing intensive treadmill exercise.

(vii) Intensive treadmill running increases the D2 receptor mRNA transcript expression but does not alter the expression of D1 receptor subtype

(viii) Preliminary data suggests that exercise has no effect on the number of SNpc neurons.

(viii) Exercise animals demonstrate decreased and normalized glutamate receptor terminal density. This finding supports but does not confirm that exercise may facilitate glutamate terminal release.

(ix) Preliminary data suggests that exercise alters GluR2 expression.

(x) Golgi staining and qRT-PCR studies have been initiated.

Component Two: Enhancement of neuroplasticity in the MPTP-lesioned nonhuman primate.

(i) The administration of the dopamine agonist Pramipexole induces dyskinesia. This occurs to a lesser degree than that observed with Sinemet. Statistically analysis is ongoing.

(ii) There is no detectable enhancement of intrinsic behavioral recovery within the first 6 weeks after MPTP-lesioning with either Sinemet or Pramipexole.

(iii) Pramipexole and Sinemet slightly increases dopamine levels in both the MPTP-lesioned mouse and MPTP-lesioned squirrel monkey. These changes are most evident in the ventral striatum.

(iv) The addition of microdialysis studies supports the neurochemical HPLC analysis. Specifically treated animals that have undergone repeated microdialysis studies demonstrate an increase in amphetamine induced dopamine release after termination of either Sinemet or Pramipexole treatment. This amphetamine effect was not seen in the MPTP –saline animals.

(v) Preliminary western blot analysis demonstrates a slight increase in TH protein expression in the dorsal caudate in Pramipexole-treated animals, but no change in DAT expression. Studies are ongoing to examine subregions of the striatum for changes in TH, DAT and VMAT protein expression both by western and immunocytochemistry techniques.

(vi) Preliminary neurophysiological studies show no difference in dopamine release using Fast-scan cyclic voltammetry. Methodological differences between microdialysis and voltammetry may explain why no increase in dopamine was observed in the drug treated groups using voltammetry.

(vi) Preliminary neurophysiological studies have demonstrated a low AMPA to NMDA ratio in normal medium spiny neurons of the nonhuman primates. After MPTP lesioning this ratio appears to diverge into two distinct AMPA/NMDA ratio characteristics. In general the population of medium spiny cells appear to diverge either to increase AMPA to NMDA ratio and another medium spiny cell type decreases AMPA to NMDA ration. These neurophysiological studies will be added to any additional nonhuman primate added to the study.

(vii) MPTP-lesioned animals demonstrate increased glutamate terminal density relative to saline treated animals. After treatment with either Sinemet or Pramipexole, there is reduced glutamate terminal density occupancy. This finding would support but not confirm that drug treatment facilitates the release of glutamate within corticostriatal terminals. Given the presence of dyskinesia in these treated animals this finding may also support the hypothesis that increased dyskinesia may be in part related to glutamate release.


(viii) q RT-PCR studies have been initiated.

Reportable Outcomes

Component 1: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.

Study 1: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.

This Aim has been completed in Year 1. A manuscript describing these studies was sent for review to *Brain Research*. However, the recent addition of new data from our electrophysiological studies (see below) and additional behavioral tests has resulted in a much stronger manuscript that we have decided to submit to the *Journal of Neuroscience*.

Briefly, an important question to be addressed in this proposal is the effect of intensive treadmill exercise in the MPTP-lesioned mouse model of basal ganglia injury on the level of striatal dopamine and its metabolites. Mice were lesioned with MPTP in a series of 4 injections at a concentration of 20 mg/kg (free-base) while another group of mice were administered saline. Mice from both the MPTP-lesioned and saline groups were subjected to either intensive treadmill exercise (1 hour per day) or no exercise for 28 days. During the exercise paradigm brain tissues were harvested at either 7 or 28 days of exercise. Striatal tissues were analyzed for dopamine and its metabolites (HVA and DOPAC) as well as the pattern of expression of protein for tyrosine hydroxylase (TH) and the dopamine transporter (DAT). Since our previous manuscript published in Year 1 of this proposal (attached as Appendix 2 of this proposal) showed that the return of DAT protein expression in the striatum is actually suppressed through intensive treadmill exercise. Another manuscript published in Year 1 of this proposal (and included as Appendix 1 in this Report) showed that there is intrinsic return of both TH and DAT protein expression 2 to 3 months after MPTP-lesioning. Initially, we expected that intensive treadmill exercise would enhance the return of these markers of basal ganglia intensity since it enhanced (accelerated) behavioral recovery (based on treadmill running duration and velocity). The suppression of DAT protein expression was in fact an unexpected outcome that we continue to pursue. Since this outcome acted as an indicator of the effect of our intensive treadmill exercise paradigm we now routinely use it to validate our intervention. The outcome of this study is that the intensive treadmill exercise paradigm does not accelerate the return of striatal dopamine. Measurement at both 7 and 28 days of exercise showed levels of striatal dopamine not to be significantly different from MPTP-lesioned non-exercise animals. Therefore, these results indicate that the enhancement of behavioral recovery in the MPTP-lesioned mouse is not apparently due to the return of striatal dopamine. Analysis of striatal glutamate using immuno-electron microscopy showed elevation in glutamate indicating that another neurotransmitter system (in this case originating from the cortico-striatal pathway) plays an important role in behavioral recovery. These results are described in a manuscript (Fisher et al 2004, Appendix 2).
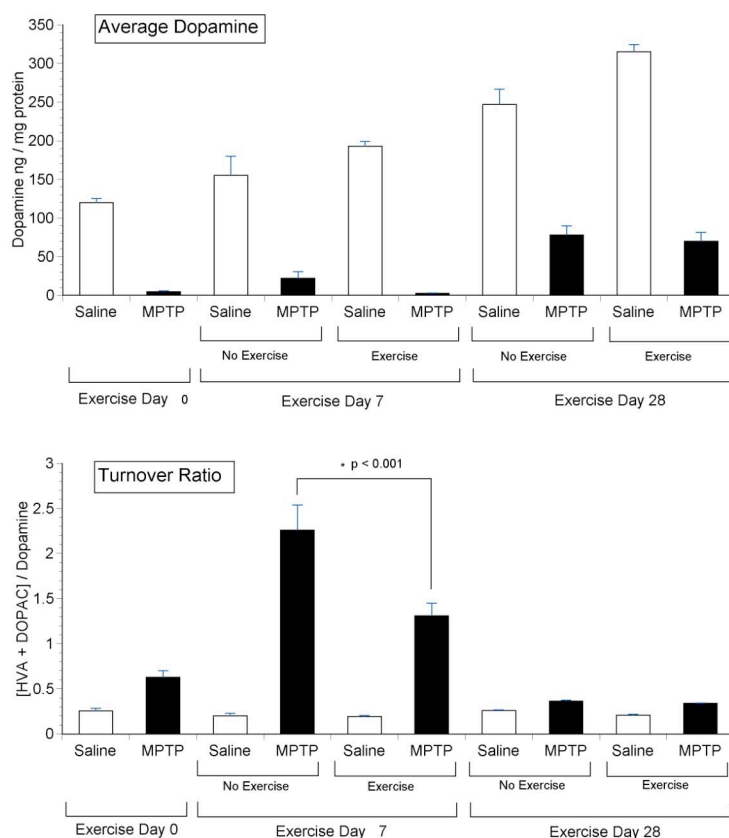
There are no deviations from Study 1.   However, to strengthen and further understand this phenomenon of alterations in dopamine with exercise, we have added two important studies: (i) fast-cyclic voltammetry; and (ii) accelerated mouse rotarod.

Our initial finding from Aim 1 is that there is no significant enhancement of the return of <u>total</u> absolute level of striatal dopamine in the MPTP-lesioned mouse with exercise, as determined using HPLC.   The HPLC technique involved the homogenization of <u>total</u> dissected striatal tissue. Measurement of dopamine using HPLC reflects the total pool of dopamine both synaptic and intracellular.   Therefore, this technique may miss alterations in intracellular levels of dopamine within remaining surviving terminals.   To address this possible alteration within intracellular dopamine storage, we established a collaboration with Dr. John Walsh to use fast-cyclic voltammetry in striatal brain slices, which measures the amount of dopamine released from terminals after stimulation. This technique is able to directly measure dopamine release from surviving dopaminergic terminals (1,2). The analysis of dopamine release were carried out in slice cultures derived from brain of mice from all four groups including (i) Saline, (ii) Saline + exercise, (iii) MPTP, and (iv) MPTP + exercise. We observed enhanced dopamine release in MPTP-lesioned animals after exercise compared compared to mice that did not go through the intensive treadmill exercise. These data are shown in Figure 2 and are included in the manuscript to be submitted to the Journal of Neuroscience.
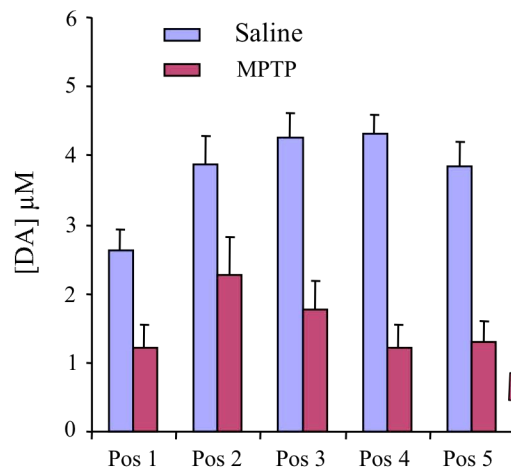
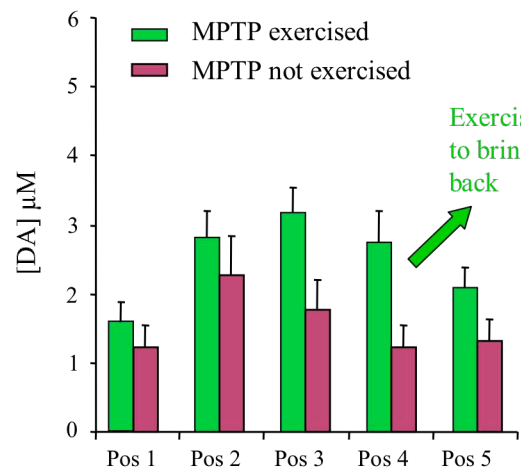## Exercise brought back the dopamine signal!
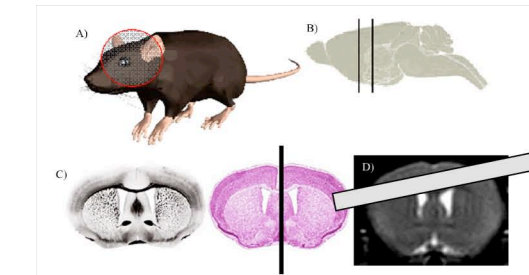
We sampled dopamine (DA) at 5 sites
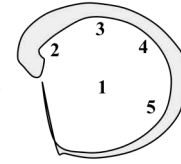
with voltammetry technique after delivering MPTP toxin one month earlier.

MPTP toxin reduced dopamine

Exercise helped to bring dopamine back

Figure 2: The summary of the electrophysiological (voltammetry studies). The graph to the left demonstrates reduced dopamine release from nigrostriatal terminals throughout the striatum after MPTP administration. The graph to the right demonstrates increased release of dopamine from surviving terminals after treadmill exercise.

In response to our critique on our manuscript submitted for review to Brain Research and the fact that we wished to extend our motor behavioral testing repertoire beyond the motorized treadmill, we added the accelerating rotarod (Columbus Instruments, Columbus, OH). Using this approach we detected differences in the ability of exercised mice (MPTP or saline) to sustain a position on the

Figure 3

Rotarod Test Results During Exercise Training
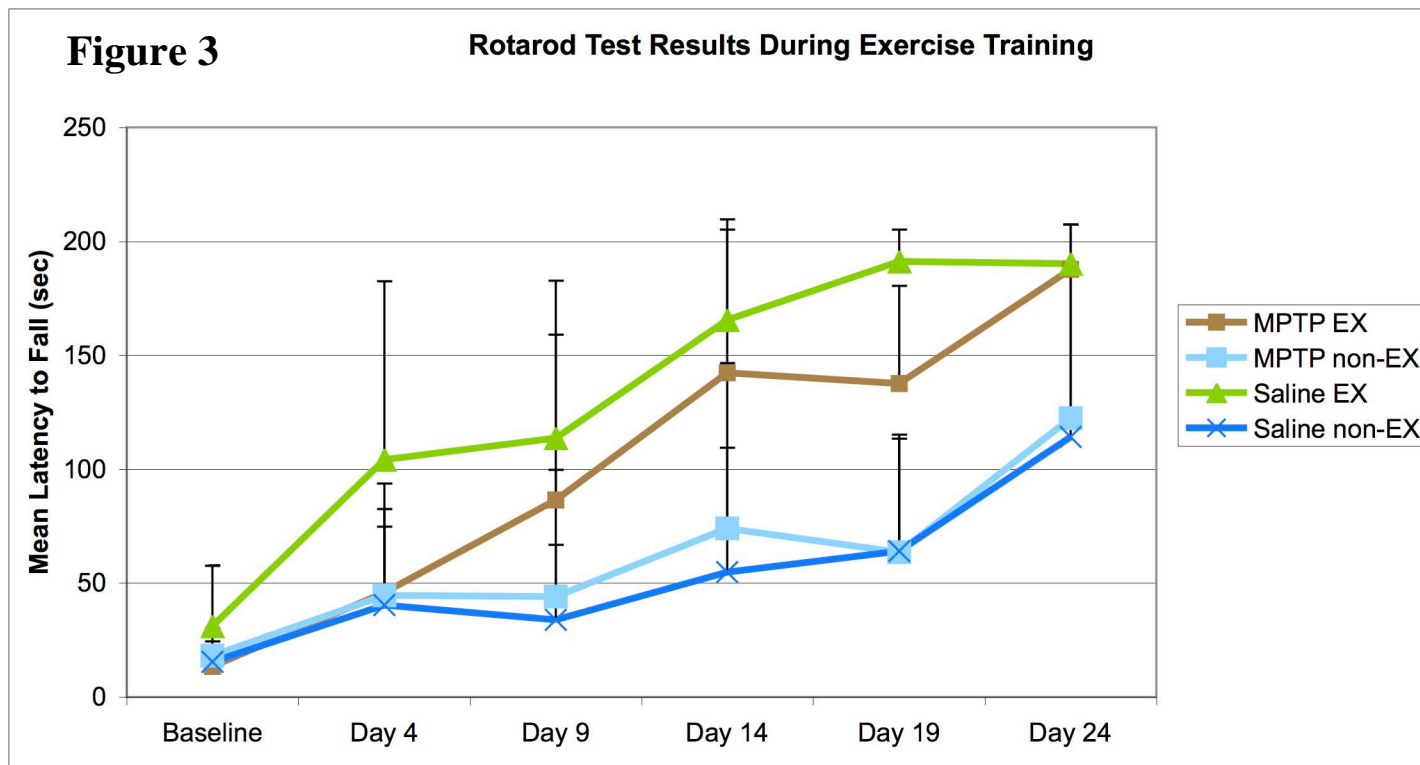
rotarod for a longer period of time compared with non-exercised mice (MPTP or saline). Mice from all groups were tested once each week for the duration of the exercise period. At the start of the exercise regimen, all groups performed the behavior test equally. However, as shown in Figure 3 the MPTP+exercise mice performed better than the MPTP-non-exercise group. This indicates that treadmill running leads to improve performance on another motor behavior task and acts as a second measure of motor behavior. These data have been included in a manuscript to be submitted to the Journal of Neuroscience.

Study 2: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine-3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, in situ hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice.

As mentioned in Study 1 (and reported in Fisher et al 2004), both striatal TH and DAT protein return after MPTP-lesioning are attenuated by intensive treadmill exercise. We now routinely use this indicator as a marker to validate the treadmill exercise paradigm. After completion of the exercise brain tissues are analyzed for the expression of striatal DAT and TH proteins comparing MPTP and

MPTP + exercise animals. Outcomes from Study 2 on the effect of exercise on DAT protein expression in the striatum are reported in a manuscript (See Fisher et al 2004, Appendix 2). As part of these studies we have analyzed the pattern of expression of TH and DAT mRNA transcripts in surviving nigrostriatal dopaminergic neurons in mice with and without MPTP-lesioning and with and without exercise. Studies were carried out using in situ hybridization histochemistry with deoxyribonucleotide probes specific for either TH or DAT. The relative degree of mRNA expression is determined by grain counting using computer assisted image analysis of emulsion-dipped sections with cell body staining. Results from this study are shown in Figure 4 following 28 days of exercise and are included in a manuscript to be submitted for review very soon. Briefly, we observed that exercise has an effect on DAT mRNA expression by down-regulating the degree of transcript expression in the surviving SNpc neurons. Down-regulation of DAT transcript may account, in part, for the down-regulation of DAT protein as previously published by our lab. Unlike DAT, exercise does not appear to significantly alter the expression of TH mRNA. The mechanisms responsible for this observation of mRNA suppression are not yet known but studies outlined in Component 1 of this proposal, which examine the administration of glutamate antagonists in exercised animals, may elucidate the role in which glutamate and its receptors play in the expression of DAT and TH.

With respect to postsynaptic effects of exercise in the MPTP-lesioned mouse, we are currently analyzing changes in the pattern of expression of CREB and DARPP-32 and their phosphorylated states using immunocytochemistry and western immunoblotting.

Figure 4: In situ hybridization histochemistry analysis of TH and DAT mRNA in midbrain nigrostriatal dopaminergic neurons.
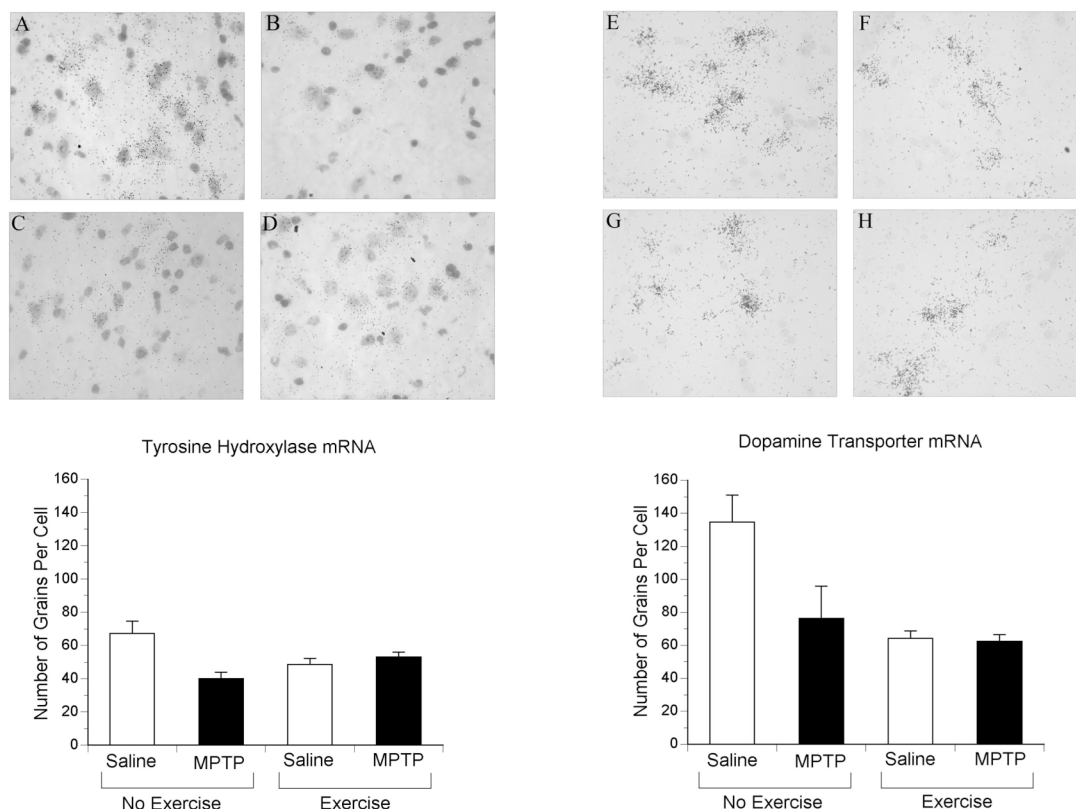
Since we observe down-regulation of DAT, we are conducting unbiased stereological cell counting of TH positive cells in all groups of animals to determine whether exercise may have a detrimental effect on remaining nigrostriatal dopaminergic neurons. Our initial findings indicate that there is no statistical significant difference in the number of TH-positive neurons between exercise and non-exercised animals either in the MPTP or saline groups. Exercise effects on DAT protein and transcript expression are therefore unlikely to be related to cell death. These findings are to be reported in the manuscript to be submitted to the Journal of Neuroscience.

Findings thus far from study 1 and study 2 suggest that while the synthesis and absolute levels of dopamine do not change with exercise, exercise does appear to effect the handling of dopamine by facilitating its release (voltammetry), and by increasing its availability in the synapse (through the down-regulation of DAT). In addition, increased dopamine released is accompanied by an increase expression of D2 receptor, but with no change in D1. One next question to address is do changes in dopamine handling, through increased phasic dopamine release alter the expression of glutamate receptors (either NMDA or AMPA), as may occur through D1 and/or D2 mediated mechanism(s)? Are alterations in glutamate receptor subtype expression mediated in part through altered DARPP-32 and CREB expression or their phosphorylated forms? These questions are currently being addressed in Study 4.

Study 3: *The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy.*

Results from this study using immuno-electron microscopy with an antibody against glutamate are presented in the published manuscript Fisher et al 2004 included as an Appendix in this report. Our published results demonstrate that exercise facilitates the release of striatal glutamate in MPTP-lesioned mouse. Given the extensive glutamatergic innervation of the striatum from the cortex, the most likely source of this glutamatergic release is from the cortico-striatal pathway. This effect appears specific to the striatum, since these changes were not observed in the hippocampus. This result provides an important foundation to carryout studies 4 and 5 in this component. Experiments will be pursued to determine if there are alterations in the pattern of expression of AMPA and NMDA receptor subunits and their phosphorylated states in the exercise paradigm. In addition Study 5 will determine if the application of AMPA or NMDA receptor antagonists will alter (1) dopamine release or handling as observed in study 1; (2) dopamine-dependent down-stream effector molecules (such as DARPP-32); (3) effect dendritic spine density, (Golgi studies).

Study 4: *The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and in situ hybridization histochemistry.*

We have initiated this study using in situ hybridization histochemistry and immunohistochemistry analysis of striatal tissues in brains of mice from all 4 groups. Probes used for mRNA transcript analysis include the AMPA receptor subunits GluR1 and GluR2 and the NMDA receptor subunits NMDA-NR1, NMDA-NR2A, NMDA-NR2B. Probes for the analysis of striatal proteins also include the previous list plus probes specific for the phosphorylated forms. Initial results from in situ hybridization histochemistry studies do not detect significant differences in the pattern of expression of AMPA receptor subunit GluR1 between all 4 groups when analyzed at the completion of

Figure 5
Example of Golgi-stained Medium Spiny Neuron from Saline No-Exercise Dosolateral Striatum
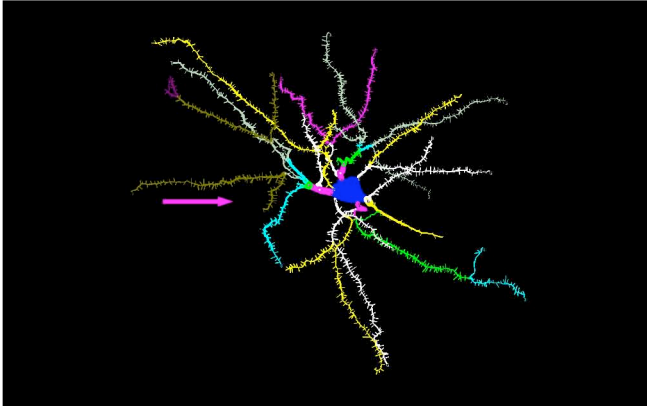
Neurolucida Cell Drawing
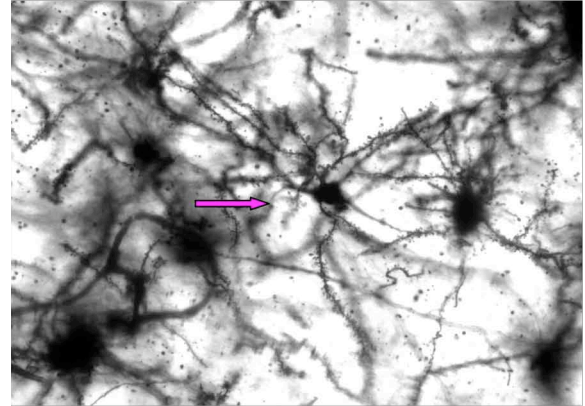
40x Medium Spiny Neuron

the exercise regimen. These tissues are continuing to be analyzed to determine if any subset of striatal neurons show alterations in transcript expression. Preliminary studies from immunohistochemistry staining suggest altered expression of AMPA receptor GluR2. This observation needs to be confirmed by both in situ hybridization histochemistry and western immunoblotting.

To further study the contribution of NMDA and AMPA receptor expression after exercise, we have added two molecular approaches, including quantitative real-time polymerase chain reaction (qRT-PCR) and Golgi staining. We added qRT-PCR to our studies to complement and confirm the in-situ studies. We have recently designed primer sets for this approach using sequences published in the literature and available through genebank data bases. We are now able to perform this technique and hope to gather meaningful data over the next several months using tissues harvested from brains of mice in all 4 groups.

In order to interpret alterations in glutamate receptor pattern of expression, we have initiated studies employing Golgi stain. Studies have shown that glutamate activation, possibly through neurotrophic factors like BDNF, may influence dendritic spine density, which may in turn influence receptor expression. This approach will allow us to determine the dendritic spine density and branching of striatal medium spiny neurons. These morphological characteristics are influenced by glutamatergic neurotransmission. We hope to gather meaningful data and determine if there are any significant differences between exercise and non-exercise MPTP-lesioned and saline mice. Figure 5 is a representation of Golgi staining in striatal tissue. This is a slight deviation from the initial aim of this proposal but it is an important complementary component in our understanding of glutamate receptor expression changes in the context of exercise. For example, this approach has been based on findings by other investigators showing morphological alterations in the hippocampus of environmentally enriched animals.

Study 5: *We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist.* After MPTP-lesioning mice will

be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning.

This Aim is designed to be pursued in Year 2.5 through 4. However, experiments addressing this Study have been initiated to validate this approach and to prepare for completion of Study 5. Both saline and MPTP-lesioned mice were administered either the AMPA receptor antagonist GYKI-52466 (0.5 or 5.0 mg/kg) or the NMDA receptor antagonist MK-801 (1 mg/kg). Mice were administered these antagonists starting 4 days after the last injection of MPTP for a period of 30 days. No adverse effects on behavior were observed. The brains from animals in all groups were harvested and analyzed for striatal expression of TH and DAT protein and for expression of TH and DAT mRNA transcripts in nigrostriatal neurons. Data from these experiments show a differential effect of using an AMPA receptor antagonist compared to an NMDA receptor antagonist. For example, comparison of the expression of TH mRNA transcripts in AMPA antagonist treated MPTP-lesioned mice show elevated TH expression in surviving nigrostriatal dopaminergic neurons.

Studies have been carried out using immuno-electron microscopy with an antibody against glutamate showing no significant alteration in glutamate synaptic occupancy in mice administered the AMPA receptor antagonists GYKI-52466 or the NMDA receptor antagonists MK-801.

To extend these studies we have collaborated with Dr. Frank Menneti at Pfizer, who has provided us with more specific AMPA and NMDA receptor antagonists targeting GluR1 and NMDA-NR2A subunits. These studies will be pursued in year 3.

Component 2: Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.
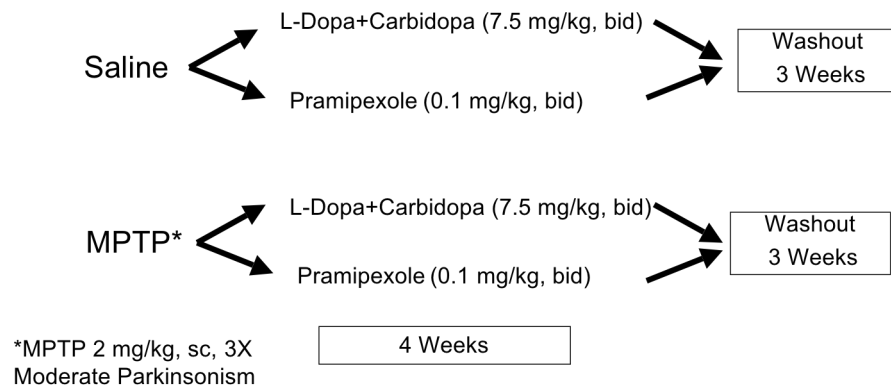
Study 1: *The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of Pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.*

In Year One of this proposal we have carried out the lesioning and behavioral analysis of MPTP-lesioned nonhuman primates in the groups utilizing the 6-week time frame. These are groups 1, 3, 5, and 7 in the Specific Aims Component 2. These squirrel monkeys were lesioned with MPTP in a series of 6 injections of neurotoxicant at a concentration of 2 mg/kg (free-base) administered once every 2 weeks for a total of 12 mg/kg per animal. The typical timeline for this protocol is outlined in Figure 1 showing that acquisition, quarantine (60 days), baseline acclimation and behavioral assessment (30 days), MPTP-lesioning (12 weeks), followed by behavioral assessment in the 6 weeks after the last injection of MPTP. The total time period for this stage is approximately 8 months.

The behavioral assessment of MPTP-lesioned squirrel monkeys with and without Pramipexole in the early (6 week) time point has been completed.   In study 1 we have added a Sinemet (L-DOPA/ Carbidopa) group for comparison with Pramipexole. This deviation is based on the scientific rationale that L-dopa, unlike Pramipexole, is metabolized and stored by dopaminergic terminals and therefore may have a more direct effect on the regulation of endogenous dopamine production and behavioral recovery and offers an interesting comparison to a compound that is not taken up by terminals.

One important outcome in Study 1 was the unexpected induction of dyskinesia in the MPTP-lesioned animals administered Pramipexole.   This new finding has not been reported in the literature by other

Drug Treatment Study: Sinemet and Pramipexole in the MPTP-lesioned Squirrel Monkey

Saline

→ L-Dopa+Carbidopa (7.5 mg/kg, bid)

→ Pramipexole (0.1 mg/kg, bid)

→ Washout 3 Weeks

MPTP*

→ L-Dopa+Carbidopa (7.5 mg/kg, bid)

→ Pramipexole (0.1 mg/kg, bid)

→ Washout 3 Weeks

*MPTP 2 mg/kg, sc, 3X
Moderate Parkinsonism

4 Weeks

investigators and we are preparing a manuscript reporting this novel finding (Figure 6). This may indicate that Sinemet and Pramipexole may both induced dyskinesias through mechanisms that include the down regulation of the dopamine transporter. Experiments to specifically address this mechanism will be carried out in years 2-3 of this proposal.

Figure 6

Prami-Sinemet Dyskinesia Study Per Dose
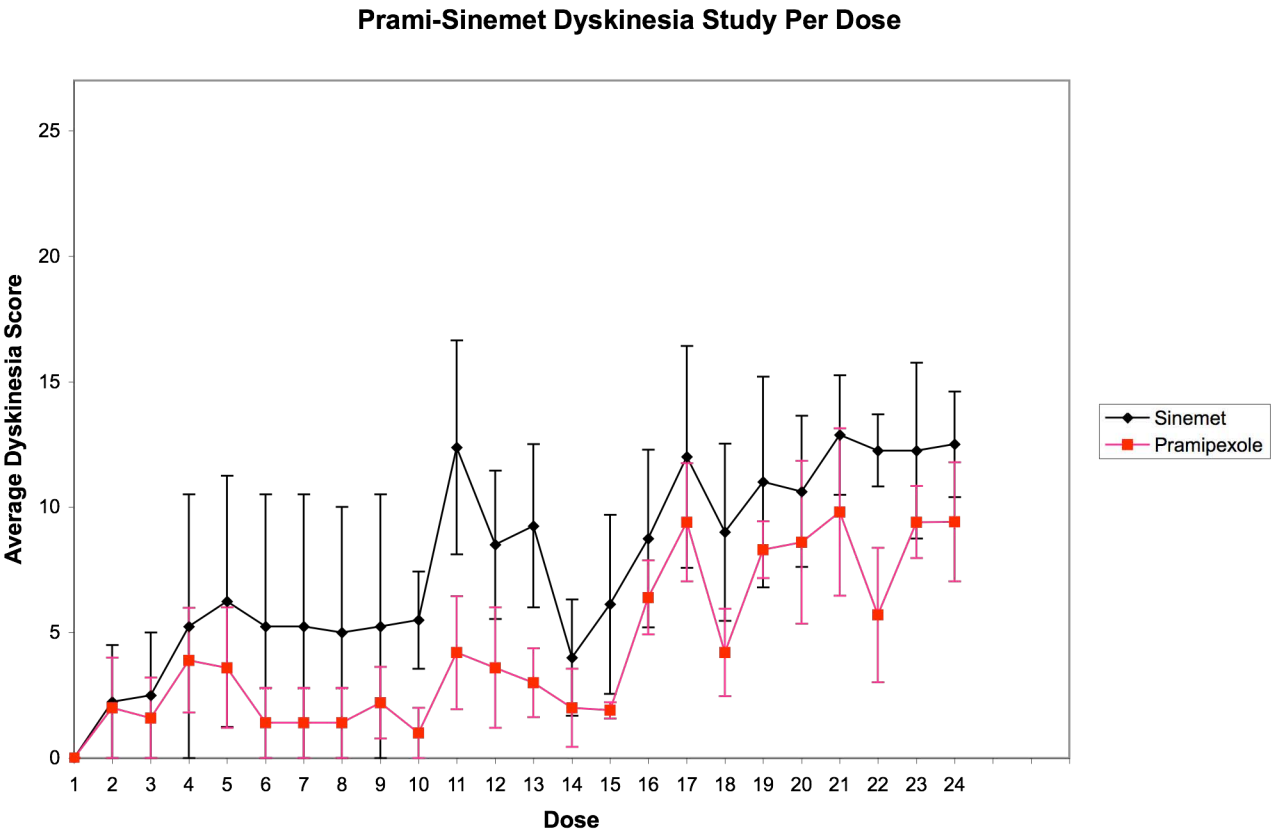
The behavioral assessment in this first group of animals carried out up to 8 weeks after the last injection of MPTP showed a slight enhancement of behavioral recovery in both the Sinemet and Pramipexole groups versus the saline treated group (Figure 7). Statistical analysis is being completed for both behavioral studies (dyskinesia and motor impairment)

Figure 7

Army Prami-Sinemet Studies Post-MPTP Clinical Ratings
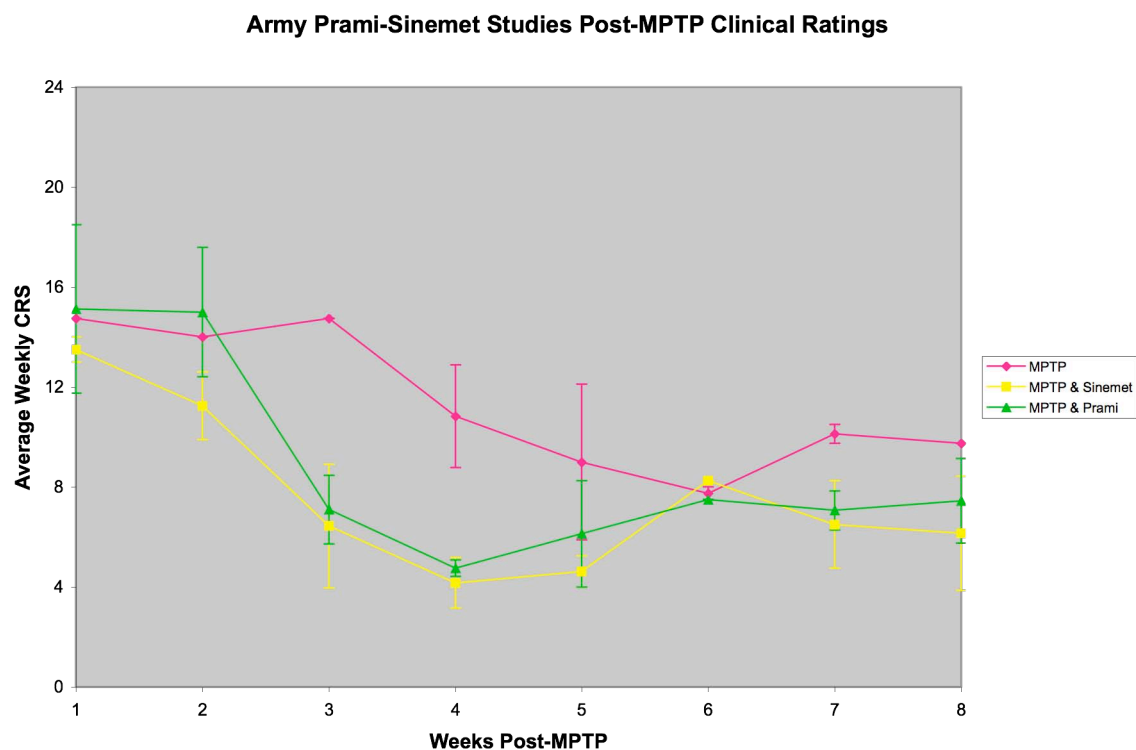
Nonhuman primates that were initially to be used for the long-term behavioral studies, were used for (1) adding a L-dopa group (2) conducting microdialysis studies to extend our findings in Study 2 (neurochemistry); and (3) to examine alterations in electrophysiolgical properties of corticostriatal neurons and voltammetry. See Sections below.

Study 2: Analysis of brain tissue from MPTP-lesioned squirrel monkeys administered Pramipexole or L-dopa/carbidopa or saline. This analysis included neurochemistry and molecular studies that examined the pattern of expression of proteins and mRNA transcripts important for dopaminergic function (including TH, DAT, VMAT2) at the level of the SNpc and CPu.

Total dopamine levels and metabolites were analyzed from all groups of animals, using HPLC. Brains were removed from all groups at completion of 4 weeks of drug or saline treatment, followed by 3 weeks of drug washout. Microdialysis of the putamen was added to this study to complement and support findings from out HPLC analysis of tissue. We found there was a slight increase in dopamine levels in the ventral caudate and putamen of animals receiving Pramipexole or Sinemet. These results are shown in Figure 8.

Figure 8: Analysis of dopamine and its metabolites in the MPTP-lesioned squirrel monkey. Squirrel monkeys were MPTP-lesioned and then treated one week after the last injection of MPTP with either Sinemet (10 mg/kg twice daily), or Pramipexole (1 mg/kg twice daily).   Animals were treated for four weeks. On each week animals received drug for three days (Tue,Wed, Thurs)) and then saline for four days (Fri, Sat, Sun, Mon). Animals were rated each day for parkinsonian features and for dyskinesia. Drug was washed out for 3 weeks and then animals were euthanized.   Brain tissue was collected and striatal tissue dissected 8 weeks (1 week monitoring + 4 weeks drug treatment + 3 weeks washout) after MPTP. HPLC analysis showed that Pramipexole and Sinemet (L-dopa + carbidopa) treated animals had a slight increase in striatal dopamine, especially in the ventral putamen, compared to MPTP + saline treated nonhuman primates.

Striatal Dopamine Levels After Dopamine Replacement Therapy
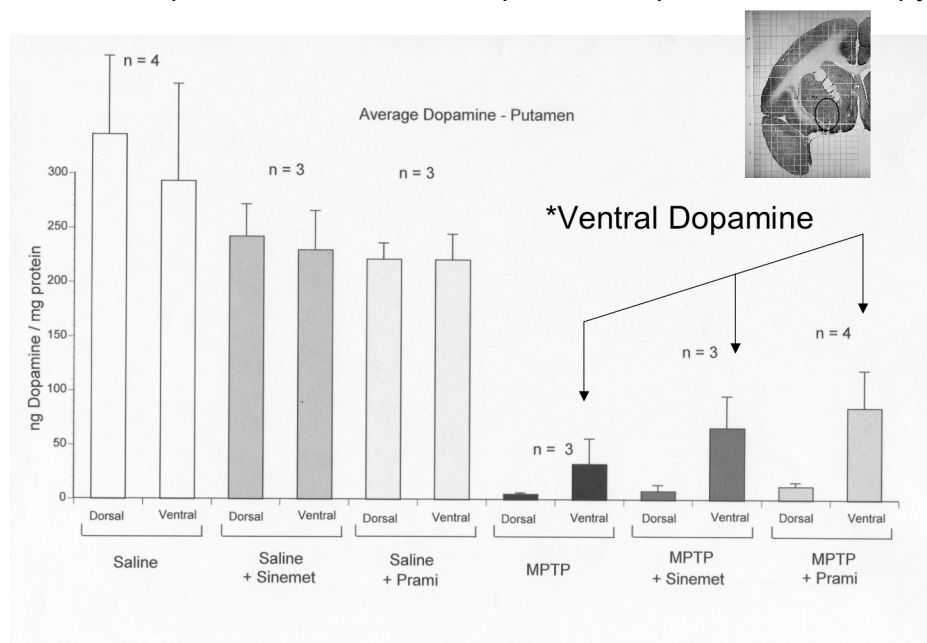
The following figure shows the timeline of microdialysis studies.

Targeting Microdialysis to Putamen of the Squirrel Monkey
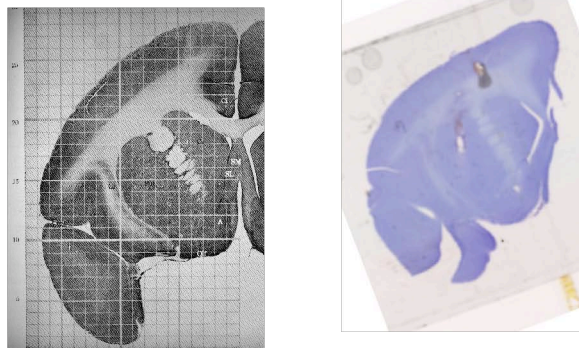
Experimental Time Line for Microdialysis

Microdialysis Sampling

MPTP
Lesioning

Drug Start   Drug Finish   Washout

4 Weeks bid   3 Weeks
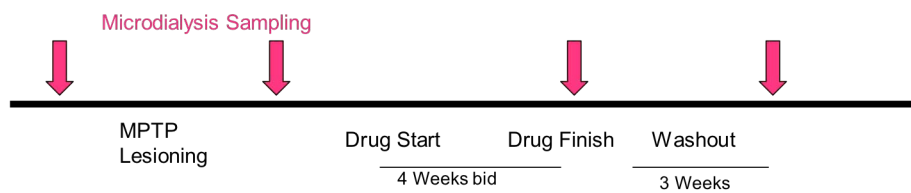
Figure 9 above shows a representative microdialysis experiment with the same animal used as its own control and undergoing repeated microdialysis studies. Our studies show that Pramipexole or Sinemet treated animals have greater amphetamine-induced dopamine release.

Dorsal Caudate TH Protein Levels after Treatment

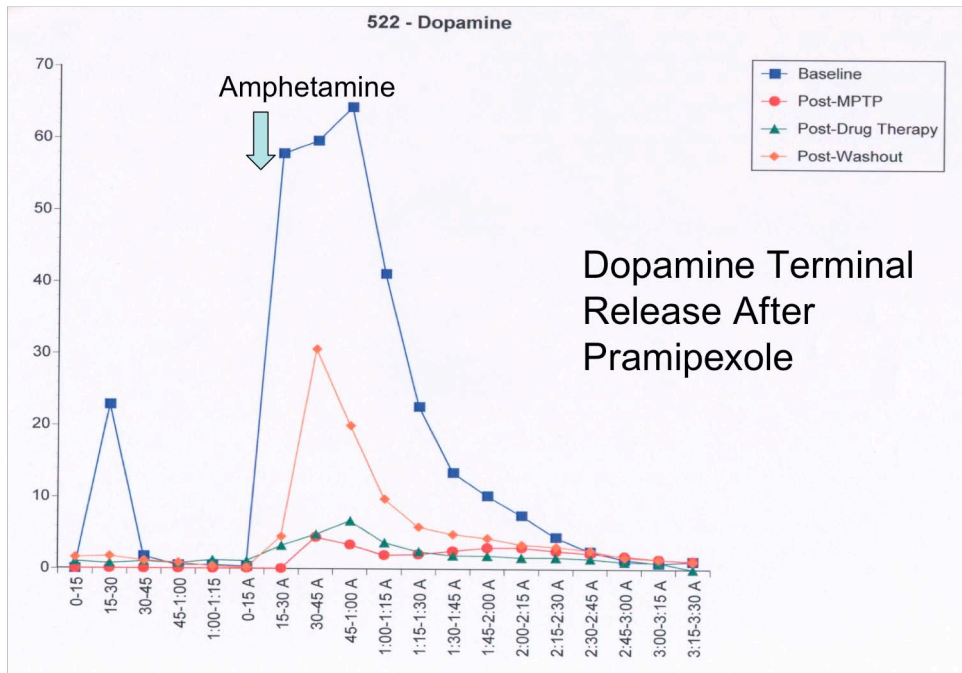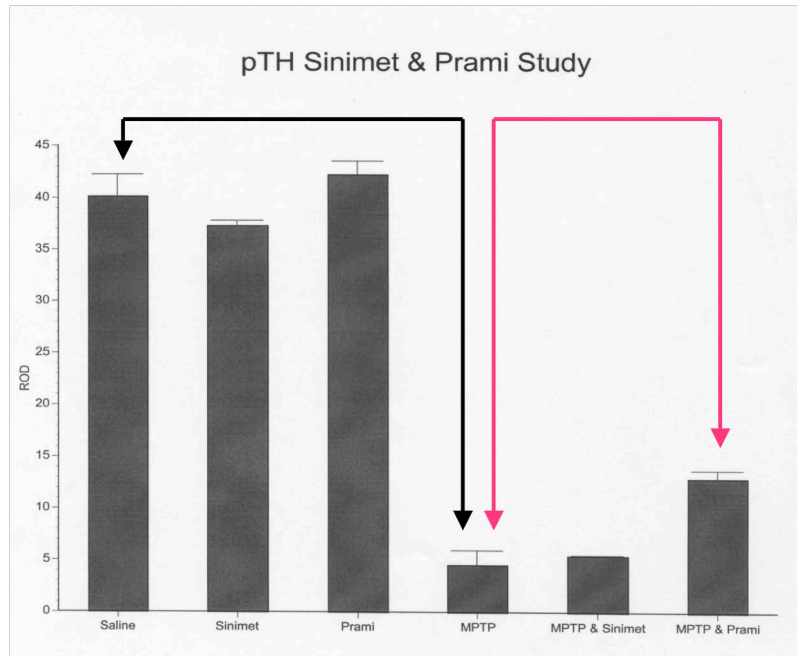**Figure 10:** Western immunoblot analysis shows that Pramipexole treated animals have a slightly grater degree of TH protein expression compared to Sinemet treated and MPTP-lesioned alone. There was no change in the pattern of expression of DAT protein in the treatment groups. Immunohistochemistry and additional western immunoblot analysis is currently underway to determine regional differences in the pattern of expression of TH and VMAT-2 in the caudate nucleus and putamen as well as within the SNpc.

We have also added physiological studies on a subset of animals to complement our current findings. Fast-scan cyclic voltammetry did not show detectable dopamine release in either the MPTP-lesioned or treated groups. The slight differences between microdialysis and voltammetry may be due to the differences in sampling where microdialysis has a greater sampling area compared to voltammetry. Electrophysiological studies also showed changes in the ratio of AMPA: NMDA contribution to the EPSPs of corticostriatal synapses after MPTP, and this ratio is altered again in treated groups.

Study 3: *The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using in situ hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate.*

To pursue this Study we have designed and tested appropriate primer sets for quantitative real-time PCR (qRT-PCR) using a newly acquired Eppendorf RT-PCR thermocycler. Brain tissues have now been collected from animals in all groups and studies including immunohistochemistry, western blotting, and in situ hybridization histochemistry will be carried out in Year 3 of this proposal.

Study 4: *The effect of Pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.*

In collaboration with Dr. Charles Meshul (Oregon Health Sciences University, Portland, OR) perfusion fixed brain tissues were harvested from a nonhuman primate from each group for analysis using immuno-electron microscopy with an antibody against glutamate. These results are summarized in the Figure below. Following MPTP-lesioning there is an increase in the relative density of striatal glutamate immunolabeling (second bar) within corticostriatal terminals. After treating MPTP-lesioned animals with Pramipexole or Sinemet the relative density of glutamate immunolabeling is reduced. Increased density of striatal glutamate within the terminal is thought to reflect decreased glutamate release. Our study would suggest that Sinemet increases glutamate release to a slightly greater extent than Pramipexole. This increased glutamate release may be one means by which dyskinesia is elicited to a greater extent in Sinemet treated animals than Pramipexole treated animals.
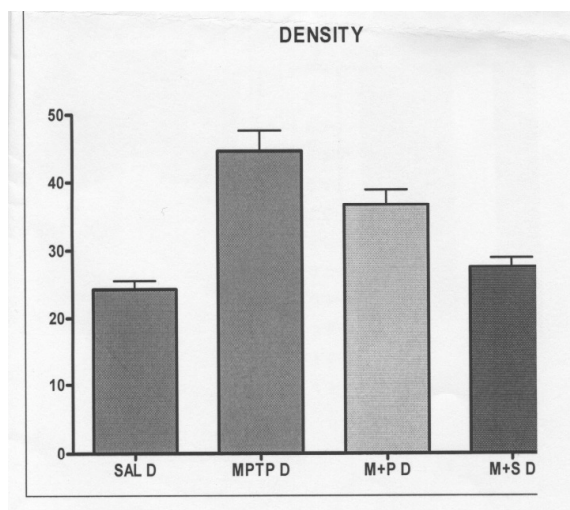
Reportable Outcomes For Years One and Two

Abstracts:

(1) Hogg, E, M. W. Jakowec, K. L. Nixon, A. T. Abernathy, P. Arevalo, B. E. Fisher, M. Liker, and G. M. Petzinger. (2004) Behavioral recovery in the MPTP-lesioned nonhuman primate: Altered dopamine biosynthesis and storage. Society for Neuroscience, San Diego, CA.

(2) Society for Neuroscience Annual Meeting, Atlanta 2006 ABSTRACT #1

Exercise induced behavioral recovery and plasticity in the MPTP-mouse model of Parkinson's disease.

Jakowec, M. W., P. Arevalo, M. Vuckovic, P. Turnquist, E. Hogg, J. Walsh[#], G. Akopian[#], C. Meshul*, A. Abernathy, M. Ramirez, B. Fisher and G. M. Petzinger.

Dept. Neurology; Davis School of Gerontology[#]; Dept. Biokinesiology and Physical Therapy; University of Southern California, Los Angeles, CA.VA Medical Center*, OHSU, Portland, OR.

The adult brain possesses a tremendous capacity for activity-dependent neuroplasticity. Following injury to the brain, physical therapy plays an important role in promoting recovery. In neurodegenerative disorders such as Parkinson's disease, physical activity improves motor function and may lead to alterations in disease progression. To better understand the role of activity-dependent plasticity in brain repair we are investigating the application of intensive treadmill exercise training in the MPTP mouse model of basal ganglia injury and dopamine depletion. Mice were administered MPTP (4 20 mg/kg each) and subjected to intensive treadmill running for 30 days starting 4 days after the last injection of MPTP (when cell death is complete) at a speed up to 20 meters/minute for 1 hour. During the exercise paradigm, mice were investigated for improvement in behavioral motor features and learning. Harvested brain tissues were analyzed by HPLC for levels of dopamine and its metabolites, and glutamate and the pattern of expression of genes and proteins for tyrosine hydroxylase, dopamine transporter, dopamine receptors D1 and D2, and AMPA and NMDA glutamate receptors using western immunoblotting, immuno histochemistry, and in situ hybridization histochemistry. Electrophysiological analysis of dopamine release was determined using fast cyclic voltammetry on brain slices. Our findings indicated that there was an enhancement of both motor behavior recovery and rotarod learning in exercised mice despite no change in the number of SNpc dopaminergic neurons and the striatal levels of dopamine. Molecular analysis showed down-regulation of DAT and TH, and significant changes in the pattern of expression of ionotropic glutamate receptors in the cortex and striatum. In addition, exercise resulted in an increase in dopamine release compared to MPTP-lesioned mice without exercise. These findings demonstrate that intensive exercise can induce dramatic neuroplasticity in an animal model of basal ganglia injury and provides a valuable framework for supporting exercise in patients with Parkinson's disease.

(3) Society for Neuroscience Annual Meeting, Atlanta 2006 ABSTRACT #2

Changes in dopamine and glutamate electrophysiology in the MPTP-treated non-human primate and the exercised MPTP-treated mouse. J Walsh*, G Akopian*, M, Jakowec[§], G, Petzinger[§]. USC Neuroscience Program, USC Davis School of Gerontology*, Department of Neurology - USC Keck School of Medicine[§].

We tested the hypotheses that dopamine (DA) and glutamate physiology are altered in the MPTP-treated squirrel monkey using electrophysiological methods. Fast cyclic voltammetry analysis of the monkey putamen revealed that MPTP treatment (6 weeks earlier) resulted in a dramatic loss in DA released in response to intra-putamen stimulation (bipolar tungsten wire electrode, 0.1 msec 100-50 µA stimulus). Saline injected monkeys showed greater DA release in the lateral versus medial putamen. To determine if excitatory amino acid receptor-mediated physiology is altered in the MPTP-treated monkey putamen we applied whole cell voltage clamp techniques and examined the relative contribution of AMPA and NMDA receptors to corticostriatal synaptic events. Saline injected monkeys showed a relatively uniform NMDA/AMPA receptor ratio, while data from MPTP-treated monkeys suggested that two new populations emerged; one with a reduced NMDA/AMPA ratio and another with an enhanced NMDA/AMPA ratio.

We applied a similar strategy to examine the impact of MPTP toxicity on DA and glutamate physiology in the mouse and, more importantly, to determine if changes striatal DA or glutamate physiology tracked the behavioral recovery induced by exercise in the MPTP-treated mouse. Cyclic voltammetry revealed a dramatic reduction in evoked DA release in the striatum of mice treated a month earlier with MPTP. A parallel group of mice were treated with MPTP and exercised daily on a treadmill. MPTP treated mice were significantly compromised in treadmill performance initially but achieved the same performance as saline injected mice by the end of one month of training. The exercise- mediated enhancement of motor skills transferred to a rotorod task. Prior work demonstrated exercise-induced suppression in striatal DAT immunocytochemistry in the MPTP-treated mouse (Fisher et al, 2004, J Neur Res 77:378), but voltammetry revealed a significant exercise-induced increase in DA release in the MPTP treated mouse.

These data demonstrate emergent dopaminergic and glutamatergic plasticity created in the striatum following exposure to the neurotoxin MPTP. We hypothesize these forms of synaptic plasticity underlie both behavioral deficits created early as well as recovery seen later in the MPTP model.

Publications:

(1) Jakowec M.W., K. Nixon, E. Hogg, T. McNeill, and G. M. Petzinger (2004) Tyrosine hydroxylase and dopamine transporter expression following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of the mouse nigrostriatal pathway. J. Neurosci. Res. **76** (4) 539-550.

(2) Fisher, B.E., G. M. Petzinger, K. Nixon, E. Hogg, S. Bremmer, C. K. Meshul, and M. W. Jakowec (2004) Exercise-Induced behavioral recovery and neuroplasticity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse basal ganglia. Journal Neuroscience Research **77**: 378-390.

(3) Jakowec, M.W., and G.M. Petzinger (2004) The MPTP-Lesioned Model of Parkinson's Disease with Emphasis on Mice and Nonhuman Primates. Comparative Medicine **54** (5) 497-513.

(4) Petzinger, GM, and M. W. Jakowec (2005) Animal Models of Basal Ganglia Injury and Degeneration and their Application to Parkinson's Disease Research. In *Parkinson's Disease*, eds M. Ebadi and R. F. Pfeiffer, CRC Press, Boca Raton, FL.

(5) Petzinger, G.M., K. Nixon, B. E. Fisher, A. Abernathy, and M. W. Jakowec. (2006) Behavioral Recovery in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned Squirrel Monkey (*Saimiri sciureus*): Analysis of Striatal Dopamine and the Expression of Tyrosine Hydroxylase and Dopamine Transporter Proteins. J. Neurosci. Res. 83: 332--347.

(6) Petzinger, G. M., and M. W. Jakowec (2006) Animal Model of Parkinson's Disease. In: Handbook of Parkinson's Disease, eds. R. Pawha and K. Lyons, Marcel Dekker, Inc., NY, NY.

(7) Petzinger, G. M., B. E. Fisher, E. Hogg, A. Abernathy, P. Arevalo, K. Nixon, A. Chua, and M. W. Jakowec (2005) Neuroplasticity and Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP)-Lesioned Mouse Model: Analysis of Striatal Dopamine, Tyrosine Hydroxylase, and Dopamine Transporter Expression. To be submitted to Journal of Neuroscience.

Presentations:

(1) Petzinger, Giselle MD " Neuroplasticity in the MPTP-lesioned Nonhuman Primate", Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb19-22, 2004.

(2) Jakowec, Michael PhD " The Role of Exercise in Enhancing Neuroplasticity in the MPTP-lesioned mouse" , Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb19-22, 2004.

(3) Petzinger, Giselle MD " Enhancing Neuroplasticity in models of Basal Ganglia Injury", Van Der Muelen Symposium, University of Southern California, Keck School of Medicine, April 1, 2005.

Conclusions:

The MPTP-lesioned mouse and squirrel monkey are valuable models for investigating neuroplasticity of the injured basal ganglia. Studies of year one from this proposal indicate that intensive treadmill exercise can enhance motor behavioral recovery and alter the time course of intrinsic neuroplasticity. Our data indicates that alterations in striatal dopamine is not the sole factor responsible for this enhanced recovery. Preliminary data supports the role of the glutamatergic system in exercise related effects on either the injured or normal basal ganglia. Therefore glutamate-dopaminergic interactions may serve as a therapeutic target for enhancing repair.

In the MPTP-lesioned primate we have not observed any enhancement of early (8 week) behavioral recovery through exogenous dopamine replacement therapy either in the form of Sinemet or

the dopamine agonist, Pramipexole.   Interestingly we have seen a modest increase in striatal (putamen) dopamine in the Pramipexole treated group. Molecular analysis are examining alterations in proteins involved in the biosynthetic pathway of dopamine.   One unexpected finding was the development of dyskinesia during treatment with Pramipexole. This behavioral finding has not been previously reported. Dyskinesia was noted to develop at a slightly less then that observed in Sinemet treated animals We believe that one possible mechanism for the development of dyskinesia in both Sinemet and Pramipexole treated animals is due to the down regulation of the dopamine transporter which may allow greater diffusion away from the synaptic cleft and a greater interaction with altered glutamate receptors. This hypothesis is currently being investigated using the same studies outlined in our proposal. Studies are underway to examine alterations in long -term behavioral recovery using dopamine replacement therapy.

Appendices: Attached.

Manuscript: Fisher B.E., G. M. Petzinger, K. Nixon, E. Hogg, S. Bremmer, C. K. Meshul, and **M. W. Jakowec**. (2004) Exercise-Induced behavioral recovery and neuroplasticity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse basal ganglia. Journal Neuroscience Research **77**: 378-390.

Manuscript: Petzinger, G.M., K. Nixon, B. E. Fisher, A. Abernathy, and **M. W. Jakowec**. (2005) Behavioral Recovery in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned Squirrel Monkey (*Saimiri sciureus*): Analysis of Striatal Dopamine and the Expression of Tyrosine Hydroxylase and Dopamine Transporter Proteins. J. Neurosci. Res. 83: 332--347.

Abstract: Society for Neuroscience, Atlanta, October, 2006

Abstract: Society for Neuroscience, Atlanta, October, 2006

References:
(1) Schulte A, Chow R.H. A simple method for insulating carbon-fiber microelectrodes using anodic electrophoretic deposition of paint. Anal Chem (1996), 68: 3054-3058

(2) Miles P.R., Mundorf M.L., Wightman M. Release and uptake of catecholamines in the bed nucleus of the stria terminalis measured in the mouse brain slice. Synapse (2002)44:188-197

Behavioral Motor Recovery in the 1–Methyl–4–Phenyl–1,2,3,6–Tetrahydropyridine-Lesioned Squirrel Monkey (*Saimiri sciureus*): Changes in Striatal Dopamine and Expression of Tyrosine Hydroxylase and Dopamine Transporter Proteins

Giselle M. Petzinger,[1,2*] **Beth Fisher,**[2] **Elizabeth Hogg,**[1] **Avery Abernathy,**[1] **Pablo Arevalo,**[1] **Kerry Nixon,**[1] **and Michael W. Jakowec**[1,2]

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The neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides an excellent opportunity to study repair and response to injury in the basal ganglia. Administration to mammals leads to the destruction of nigrostriatal dopaminergic neurons and depletion of striatal dopamine. In the squirrel monkey (*Saimiri sciureus*), MPTP-lesioning results in parkinsonian motor symptoms including bradykinesia, postural instability, and rigidity. Over time animals display motor behavioral recovery. To better understand this mechanism we employed a lesioning regimen of two or six subcutaneous injections of MPTP (2.0 mg/kg, free-base) to generate mild or moderate parkinsonism. Brain tissue was harvested at 6 weeks or 9 months after the last injection and analyzed for dopamine and its metabolites by high performance liquid chromatography (HPLC), and by immunohistochemical staining and Western immunoblotting for the expression of tyrosine hydroxylase (TH), dopamine transporter (DAT), and dopamine- and cAMP-responsive protein phosphatase of 32 kDa (DARPP-32), an effector molecule enriched in striatal medium spiny neurons. Several months after MPTP-lesioning, when squirrel monkeys displayed full motor behavioral recovery, striatal dopamine levels remained low with a greater return in the ventral striatum. This finding is consistent with other reports using neurotoxicant-lesioning models of the basal ganglia in rodents and other species of nonhuman primates. Elevated dopamine turnover ratio and decreased DAT expression appeared in early behavioral recovery at the 6-week time point in both mild- and moderate-parkinsonian monkeys. Tyrosine hydroxylase and DAT expression was increased in late stage recovery even within dopamine-depleted regions and supports sprouting. Altered DARPP-32 expression suggests a role of medium spiny neurons in recovery. © 2005 Wiley-Liss, Inc.

Key words: basal ganglia; Parkinson's disease; nonhuman primate; neuroplasticity

The neurotoxicant 1-methyl–4-phenyl–1,2,3,6-tetrahydropyridine (MPTP) provides an excellent opportunity to study repair and the response to injury in the basal ganglia. MPTP is a meperidine derivative that can be administered systemically, and the pre-toxin form crosses the blood–brain barrier and is converted to the toxic form 1-phenyl–4-phenylpiperidium (MPP[+]) by astrocytic monoamine oxidase B (Chiba et al., 1985). MPP[+] acts as a "false–substrate" for the dopamine transporter (DAT) and accumulates in dopaminergic neurons where it targets mitochondrial complex I leading to energy depletion and the formation of reactive oxygen

species (Dauer and Przedborski, 2003). This results in the selective destruction of nigrostriatal dopaminergic neurons and the depletion of the striatal neurotransmitter dopamine similar to that seen in Parkinson's disease (PD) (Jakowec and Petzinger, 2004). Dopamine depletion results in behavioral motor deficits in mice and nonhuman primates. Motor behavioral changes in mice tend to be subtle and require specific behavioral testing including treadmill, paw-reach, and rotorod balancing to become evident (Sedelis et al., 2000, 2001; Tillerson et al., 2001; Fisher et al., 2004). In nonhuman primates, motor behavioral deficits resembling those seen in humans with PD or drug addicts who self-administered MPTP are evident and include akinesia, bradykinesia, postural instability, freezing, and in some species a resting tremor (Burns et al., 1983; Langston et al., 1983, 1984).

MPTP has been administered to a variety of different nonhuman primates using several different regimens. In our laboratory we utilize the squirrel monkey (*Saimiri sciureus*), a New World monkey. We have developed a lesioning regimen that consists of either a series of two or six injections of MPTP at a concentration of 2.0 mg/kg (free-base) with 2 weeks between injections. With this lesioning regimen we avoid high animal mortality often experienced with MPTP use. Behavioral analysis using a clinical rating scale (CRS) we designed specifically for the MPTP-lesioned squirrel monkey, documents either mild (two injections) or moderate (six injections) parkinsonian motor behavior. Interestingly, over time, animals display motor recovery. By 6 weeks post-MPTP, animals show partial motor recovery and by 9 months post-MPTP both groups of monkeys show motor behavior that is indistinguishable from nonlesioned animals. Specifically, recovered animals no longer display akinesia or bradykinesia and show normal spontaneous movement (climbing and jumping), normal hand dexterity, and balance. Motor behavioral recovery has been observed in several other species of nonhuman primates including the marmoset, vervet, and macaque (Rose et al., 1989b; Rothblat and Schneider, 1994; Schneider et al., 1998; Elsworth et al., 2000). It has been shown that motor behavioral recovery takes place with the incomplete return of striatal dopamine. Neurotoxicant lesioning of the dopaminergic system in rodents, cats, and nonhuman primates suggest that there are pre- and post-synaptic alterations within remaining dopaminergic neurons and their targets, respectively, that may compensate for the deficient return of striatal dopamine and may account for motor behavioral recovery. Presynaptic indices include increased dopamine synthesis, turnover, and release, altered dopamine uptake, and sprouting of surviving dopaminergic neurons (Zigmond et al., 1990). Alterations in dopamine synthesis may be reflected by either an increase in the activity or total protein level of tyrosine hydroxylase (TH), the rate limiting enzyme of dopamine biosynthesis. In addition, alteration in dopamine uptake may reflect the level of dopamine transporter (DAT) protein because DAT is a key component in regulating dopamine synaptic occu-

pancy (Gainetdinov et al., 2002). Post-synaptic indices include altered dopamine receptor number and function and their influence on neuropeptide expression, second messengers, transcription factors, and down-stream effector molecules especially within striatal medium-spiny neurons (Greengard et al., 1999; Gerfen, 2000; Vallone et al., 2000).

This study examines the relationship between motor behavioral recovery and pre- and post-synaptic indices of dopaminergic function, including striatal dopamine in the MPTP-lesioned squirrel monkey. We examined squirrel monkeys that were rendered either mildly or moderately parkinsonian and during early and late periods of recovery. Specifically, squirrel monkeys were administered MPTP as a series of either two or six injections 2 weeks between injections to generate either a mild or moderate parkinsonian group, respectively. Brain tissue was harvested at 6 weeks or 9 months after the last injection of MPTP. For neurochemistry and behavior, analyses were designed to compare individual groups. Molecular studies focused on the expression of two important pre-synaptic proteins, tyrosine hydroxylase and the dopamine transporter. As an initial approach to investigate post-synaptic changes, the pattern of expression of dopamine- and cAMP-responsive protein phosphatase of 32 kDa (DARPP-32), a molecule important for the down-stream action of dopamine neurotransmission in striatal medium spiny neurons, was examined. For molecular studies, analyses were designed to compare changes in the mild-lesioned group (two injections of MPTP) at the early and late time point, and changes in the moderate-lesioned group (six injections of MPTP) at the early and late time point.

MATERIALS AND METHODS

Animals

Twenty-four young–adult male squirrel monkeys (*Saimiri sciureus*) weighing 900–1,200 g each were used in these studies (Osage, St. Louis, MO). All procedures utilizing the nonhuman primate strictly followed guidelines set forth by the National Institutes for Health for the humane treatment of animals in research and had the approval of the University of Southern California Institutional Animal Care and Use Committee (IACUC). Animals were housed individually in a home cage. After quarantine, animals were acclimated to the facility for 30 days before behavioral analysis in their home cages.

MPTP-Lesioning

MPTP (Sigma, St. Louis, MO) was administered in a series of subcutaneous injections set 2 weeks apart at a concentration of 2.0 mg/kg (free-base) dissolved in sterile water and made up fresh from a new 100-mg bottle each time. One group of animals ($n = 8$) received a series of two injections (for a total of 4.0 mg/kg MPTP, free-base). Another group of animals ($n = 8$) received a series of six injections of MPTP (for a total of 12.0 mg/kg, free-base). A saline injected group ($n = 8$) acted as control. Animals were divided into the following six groups with $n = 4$ per group: (1) saline-injected

TABLE I. Parkinsonian Clinical Rating Scale Modified for Squirrel Monkey

Parkinsonian motor feature	Rating
1. Spatial hypokinesia (movement around cage)	0- Normal (uses entire cage space) 1- Utilizes most of the cage (at least 75% of cage space), but may be slow. 2- Definitely slowed, but uses more than 50% of cage space. 3- Definitely slowed, using less than 50% of cage space. 4- Does not move from a confined area, with little or no movement
2. Body bradykinesia	0- Normal movement around cage or bars 1- Slow or deliberate body movements, could be normal for age. 2- Moderately slow, intermittent limb dragging, moves without provocation. 3- Marked slowness, requires provocation to move arms or legs. 4- Frozen, little or no body movements regardless of provocation.
3. Manual dexterity (right arm)	0- Normal 1- Mildly slow or some loss of maneuverability of food items, could be normal for age. 2- Moderate slowness, noticeable effort needed to grab or maneuver food. 3- Marked slowness, with multiple attempts needed to grab food, may use both hands, may drop food. 4- Severe slowness, with inability to grab or maneuver food, may need to be hand fed.
4. Manual dexterity (left arm)	0- Normal 1- Mildly slow or some loss of maneuverability of food items, could be normal for age. 2- Moderate slowness, noticeable effort needed to grab or maneuver food. 3- Marked slowness, with multiple attempts needed to grab food, may use both hands, may drop food. 4- Severe slowness, with inability to grab or maneuver food, may need to be hand fed.
5. Balance	0- Normal 1- Slight tendency to hold on to cage, may be normal for age or no falls 2- Uses both hands intermittently for support or rare occasional falls 3- Uses both hands for support at all times or frequent falls 4- Continually hanging on for support or falls with any attempt to move
6. Freezing (observation over 4 minute clinical evaluation)	0- None, no freezing ever observed 1- Occasional mild (<5 sec) freezing episodes 2- Mild freezing episodes <5 sec duration or rare severe (>5 sec) 3- Frequent severe freezing observed >5 sec 4- Frozen most of the time

harvested at 6 weeks; (2) saline-injected harvested at 9 months; (3) MPTP-injected 2-times harvested at 6 weeks after the last injection; (4) MPTP injected 2-times harvested at 9 months after the last injection; (5) MPTP-injected 6-times harvested at 6 weeks after the last injection; and (6) MPTP-injected 6-times harvested at 9 months after the last injection.

Clinical Rating Scale for Motor Behavioral Analysis

Behavioral motor features were determined using a cage-side clinical rating scale (CRS) based on the Unified Parkinson's Disease Rating Scale (UPDRS) and modified for the squirrel monkey. Table I outlines the features of the clinical rating scale. The CRS consisted of six items including: (1) spatial hypokinesia (movement around cage); (2) body bradykinesia; (3) manual dexterity in left arm; (4) manual dexterity in right arm; (5) balance; and (6) freezing. Each item has a score of 0–4 resulting in a maximum motor score deficit of 24 points. A CRS score of <4 points is within the range of normal. Two investigators, blinded to the animal and treatment group, assessed motor features three times per week in the morning before feeding time. Baseline behavior was determined in the 2-week period before the first injection of MPTP or saline. Parkinsonian motor features using the CRS were first determined 2 weeks after the last injection of MPTP or saline, and continued on a weekly basis until tissue harvesting at 6 weeks or 9 months. Data for statistical analysis were carried out at baseline and post-injection time points of

2 weeks, 6 weeks, and 9 months. At each of these four time points a nonparametric Kruskal–Wallace analysis with Mann-Whitney post-hoc was carried out comparing saline, 2-time, and 6-time injected groups.

Brain Tissue Harvest and Preparation

Brain tissue was harvested from four animals in each group at either 6 weeks or 9 months after the last injection of MPTP for a total of 24 animals. Animals were sedated with ketamine (0.3 ml of 100 mg/ml) followed by a lethal dose of sodium pentobarbital (2 ml of 50 mg/ml solution) and monitored for eye reflex, breathing, and heartbeat. On cessation of vital signs, brains were quickly removed, briefly cooled on wet ice, and sectioned at 3-mm thickness in the coronal plane using an acrylic brain block designed specifically for the squirrel monkey brain starting at a position approximately 3 mm rostral to the midbrain. This resulted in six rostral coronal slices of 3-mm thickness. One fresh slice through the mid-striatum (at level A15–A12 in the anterior–posterior plane) of each animal was used to free-hand dissect out the entire caudate nucleus or putamen, sectioned into four pieces in the dorsal–ventral plane, and snap frozen on dry ice to be used for either high performance liquid chromatography (HPLC) analysis of dopamine and its metabolites or protein analysis using western immunoblotting (Emmers and Akert, 1963). The remaining caudal brain, which included the midbrain, was split at the mid-plane into two equal halves. Brain slices

were immersion fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) for 48 hr, cryoprotected in 20% sucrose in phosphate buffer (PB) (pH 7.4) for 24 hr, and frozen onto microscope slides using immersion into isopentane on dry ice. For immunohistochemical staining, tissue was cut at 30-μm thickness using a Leica 1950 cryostat. Sections were placed free-floating in PBS (pH 7.4) containing 0.01% thimerosal and refrigerated until used.

HPLC Analysis of Dopamine and Its Metabolites

Neurotransmitter concentrations were determined according to an adaptation of Irwin et al. (1992) of the method of Kilpatrick et al. (1986). Tissues for analysis were homogenized in 0.4 N perchloric acid and centrifuged at $12,000 \times g$ to separate precipitated protein. The protein pellet was resuspended in 0.5 N NaOH and the total protein concentration determined using the Coomassie Plus protein assay system (Pierce, Inc., Rockford, IL) using a Biotek Model Elx800 microplate reader and the software KCjunior. The concentrations of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), were assayed by HPLC with electrochemical detection (ECD). Samples were injected with an ESA autosampler. Dopamine and its metabolites were separated by a 150 mm $\times$ 3.2 mm reverse phase 3-μm diameter C-18 column (ESA, Chelmsford, MA) regulated at 28°C. The mobile phase MD-TM (from ESA) consisted of acetyl nitrile in PB and an ion–pairing agent delivered at a rate of 0.6 ml/min. The electrochemical detector was an ESA model Coularray 5600A with a 4-channel analytical cell with set potentials at -100 mV, 50 mV, and 220 mV. The HPLC was integrated with a Dell GX-280 computer with analytical programs including ESA Coularray for Windows software and the statistics package InStat (San Diego, CA). For neurochemistry, four separate basal ganglia regions were analyzed (ventral putamen, dorsal putamen, ventral caudate, or dorsal caudate) using multiple one-way ANOVA. Data were compared from the following six groups: (1) saline-injected harvested at 6 weeks; (2) saline-injected harvested at 9 months; (3) MPTP-injected 2-times harvested at 6 weeks after the last injection; (4) MPTP injected 2-times harvested at 9 months after the last injection; (5) MPTP-injected 6-times harvested at 6 weeks after the last injection; and (6) MPTP-injected 6-times harvested at 9 months after the last injection. Independent t-test showed no differences between the saline groups for each of the independent measures and as such, the two saline groups were collapsed into a single group for further analysis. Post-hoc contrasts with Bonferroni correction were carried out to determine the locus of any significant differences. For all statistical tests significance was set at $P < 0.05$.

Stereologic Analysis of Nigrostriatal Neurons

To verify the degree of lesioning in the 2-time and 6-time injection groups at the time of harvest (9 months) the number of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNpc) was determined using unbiased stereology with the computer-imaging program BioQuant (BioQuant Image Analysis Inc., Nashville, TN) on a Dell computer and an Olympus BX-50 microscope equipped with a motorized stage and Retiga CCD camera. The total number of SNpc dopaminergic neurons was determined based on the method of Gunderson and Jensen (1987). The hemi-sectioned brain (total of six animals: two each from saline, 2-time, and 6-time MPTP-injected collected at 9 months after the last injection of MPTP) with its most rostral sections starting at level A9.0 based on the brain atlas for the squirrel monkey (Emmers and Akert, 1963) was mounted in the cryostat at a temperature of -18°C. Tissue was sliced at 30-μm thickness and every sixth section collected starting just before the rostral aspect of the substantia nigra (at level A8.0) completely through to the most caudal aspect when emergence of the pontine nucleus (at level A3.5) occurs. Tissue sections were stained for TH-immunoreactivity and counterstained for Nissl substance. The SNpc was delineated from the rest of the brain based on TH-immunoreactivity of remaining SNpc neurons because the MPTP-lesioning regimen of two or six injections used in these studies leads to 30–40% of remaining TH-immunoreactive dopaminergic neurons (in the case of the 6-time MPTP injections regimen) compared to saline (see Fig. 3). In addition, anatomic landmarks at the rostral and caudal aspects of the SNpc were also used for delineation including the cerebral peduncle, third nerve, and pontine nucleus as described in the atlas for the squirrel monkey (Emmers and Akert, 1963). Each stained ventral mesencephalon section was viewed at low magnification (10× objective) and the SNpc outlined and delineated from the ventral tegmental-immunoreactive neurons using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at high magnification (80× objective) and counted if they displayed TH-immunoreactivity and had a clearly defined nucleus, cytoplasm, and nucleolus.

Immunohistochemical Analysis of Striatal TH, DAT, and DARPP-32

The antibodies used in these studies were all purchased from Chemicon, Inc. (Temecula, CA) and included anti-tyrosine hydroxylase (polyclonal made in rabbit or monoclonal made in mouse), anti-dopamine transporter protein (monoclonal made in rat), and anti-DARPP-32 (polyclonal made in rabbit). Sections were rinsed in TBS (50 mM Tris pH 7.4 with 0.9% NaCl), exposed to primary antibody (concentration of 1:1,000) for 48 hr at 4°C, rinsed in TBS, and then exposed to secondary antibody made against the species of the primary antibody (ABC Elite Kit, Vectastain, Burlingame, CA). Antibody staining was visualized by development in diaminobenzoic acid/H_2O_2. To ensure that differences in staining intensity were due to differences in antigen expression, multiple sections from each treatment group and time point were carried out concurrently under identical staining conditions. Specificity of antibody probes was verified by methods that eliminated staining including: (1) omitting primary antibody; (2) omitting secondary antibody; or (3) omitting both primary and secondary antibodies. The relative intensity of labeling between stained sections in the different treatment groups was determined using computer assisted image analysis. Slides were simultaneously scanned on a high-resolution transmission light scanner (UMAX Powerlook 1120) and digitized images analyzed using NIH Image (version 1.66). Slide and section background (using a region of interest through the corpus cal-

losum) were captured. Because sections for comparison were stained simultaneously within an experiment, variability in background was minimal. Comparison of immunostaining based on the relative optical density was determined by analyzing images using a region of interest within the quadrant of the dorsal putamen (for DAT and DARPP-32 analysis). The dorsal putamen was selected for analysis because it plays a prominent role in motor behavior. At least six to eight sections from two or three animals were used for analysis. For statistical analysis, a one-way ANOVA comparing the individual groups was carried out. The saline groups were collapsed because an independent t-test showed no differences between the saline groups for each of the independent measures. Post-hoc contrasts with Bonferroni correction were carried out to determine the locus of any significant differences. For all statistical tests significance was set at $P < 0.05$.

Western Immunoblot Analysis of Striatal TH and DAT

Western blotting was used to determine the relative protein expression of TH and DAT in the basal ganglia. The immunoblotting technique was described previously (Jakowec et al., 1995). Tissue dissected from the dorsal putamen was homogenized in buffer (25 mM Tris pH 7.4, 1 mM EDTA, 100 μM PMSF, 0.1% SDS). The dorsal putamen was selected for analysis because it plays a prominent role in motor behavior. Protein concentration was determined by the BCA method (Pierce, Inc., Rockford, IL). Equal amounts of protein (25 μg) were separated by the method of Laemmli (1970). Proteins were transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al., 1979). Filters were blocked in TS-Blotto (50 mM Tris pH 7.4, 0.9% NaCl, 5% non-fat milk), then primary antibody (1:2,000), exposed to secondary antibody and visualized by chemiluminescence (Pierce, Inc.). Filters were apposed to film (Hyperfilm ECL, Amersham, Inc.) and processed in X-OMAT developer. Images were scanned into a computer using a Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA) and the intensity of bands determined using computer assisted image analysis (NIH Image). The intensity of bands from Western blot autoradiographs was expressed as relative optical density with the saline injected group normalized to 100% because there was no significant difference between 6 weeks and 9 months. For statistical analysis, a one-way ANOVA comparing the individual groups was carried out. The saline groups were collapsed because an independent t-test showed no differences between the saline groups for each of the independent measures. Post-hoc contrasts with Bonferroni correction were carried out to determine the locus of any significant differences. For all statistical tests significance was set at $P < 0.05$.

RESULTS

Motor Behavioral Deficits After MPTP-Lesioning and the Time Course of Motor Behavioral Recovery

Motor behavior was assessed using a Clinical Rating Scale (CRS) as outlined in Table I and results shown in Figure 1. The baseline CRS scores before saline or MPTP administration for all groups of animals ranged
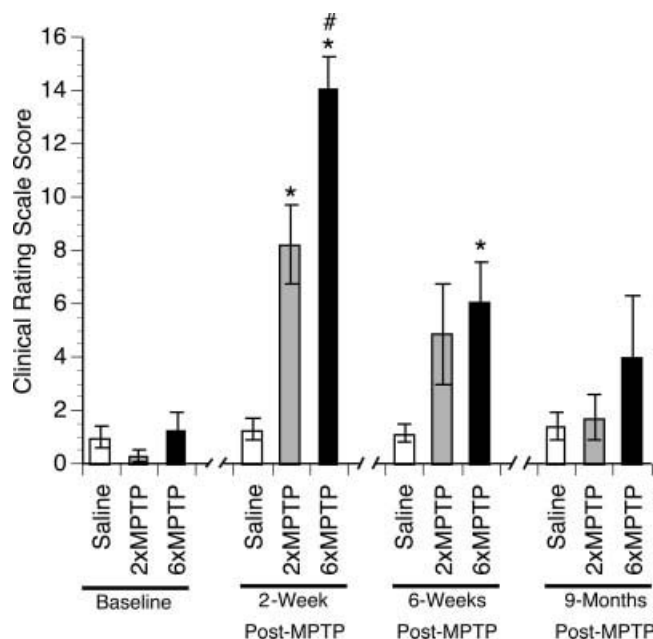

Fig. 1. Time course of motor behavior using the CRS. Baseline behavior was determined in the 2-week period before the first injection of MPTP or saline. Parkinsonian motor features using the CRS were first determined 2 weeks after the last injection of MPTP or saline, and continued on a weekly basis until tissue harvesting at 6 weeks or 9 months. Data for statistical analysis were carried out at baseline and post-injection time points of 2 weeks, 6 weeks, and 9 months. At each of these four time points a nonparametric Kruskal-Wallace analysis with Mann-Whitney post-hoc was carried out comparing saline, 2-times, and 6-times injected groups. *Indicates statistical significance compared to the saline CRS score at $P < 0.05$. #Indicates statistical significance compared to the 2-times MPTP-injected group CRS score at $P < 0.05$.

from 1–2 of a total score of 24. Throughout the entire study, saline-injected animals had a CRS score of 1.2 ± 0.2. After completion of MPTP administration, all lesioned animals showed parkinsonian features including akinesia, bradykinesia, postural instability, and freezing. Two weeks after the last injection of MPTP, animals that received two injections of MPTP were mildly parkinsonian (8.3 ± 1.2), whereas animals that received six injections of MPTP were moderately parkinsonian (14.1 ± 1.2). The difference between the saline, 2-time, and 6-time MPTP-injected groups 2 weeks after the last injection of MPTP was significant At 6-weeks post-injection, there remained a significant differences between groups ($\chi^2 = 5.40$, $df = 2$, $P < 0.05$). The 6-time MPTP-injected group remained significantly more parkinsonian than the saline group (CRS of 6.1 ± 1.5 and 1.2 ± 0.2, respectively, $P < 0.014$). The 2-time MPTP-injected group was different than the saline group but did not reach significance (CRS of 4.9 ± 1.9 and 1.2 ± 0.2, respectively, $P < 0.2$). At the 9-month time point, both the 2-time and 6-time MPTP-injected groups displayed complete motor behavioral recovery (CRS score
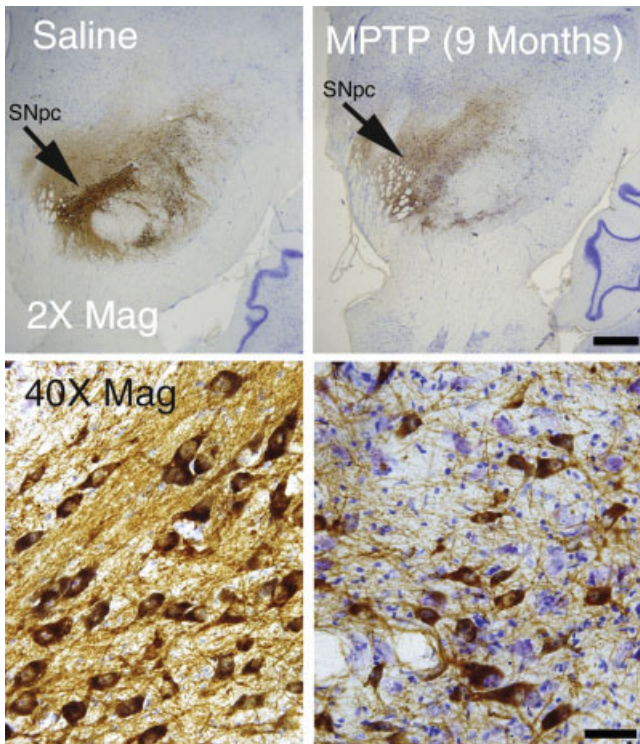
Fig. 2. Immunohistochemical staining for TH protein in the SNpc. Representative sections are shown through the mid-SNpc immunostained for TH and Nissl substance comparing saline-injected and 6-time MPTP-injected groups 9 months after the last injection. **Upper panels:** Photomicrographs at low magnification. **Lower panels:** Photomicrographs at high magnification. Note the severe depletion of TH-immunoreactive cells in the SNpc in the MPTP-lesioned SNpc despite the complete return of motor behavior. Scale bar = 800 μm (upper panels); 40 μm (lower panels). SNpc, substantia nigra pars compacta.

of 1.8 ± 0.9 and 4.0 ± 2.3, respectively) and were not significantly different from the saline group.

Stereologic Counting of SNpc Dopaminergic Neurons

Unbiased stereologic counting methods were used to determine the degree of MPTP-lesioning based on number of TH-immunoreactive midbrain neurons at the 9-month time point. Saline animals had a total of $59,100 \pm 4,440$ TH-immunoreactive midbrain neurons, whereas the 2-time MPTP-injected group had 64.5% depletion (to $21,000 \pm 2,500$), and the 6-time MPTP-injected group had an 81% depletion in cell number (to $11,300 \pm 900$). Figure 2 is a representative image staining for TH and Nissl in the mid SNpc showing the large degree of dopaminergic cell loss even at 9 months after the last injection of MPTP in the 6-time injection group.

HPLC Analysis of Dopamine and Its Metabolites

HPLC analysis of dopamine and its metabolites (HVA and DOPAC) was carried out on the dorsal puta-

men, dorsal caudate nucleus, ventral putamen, and ventral caudate nucleus of at least 3 animals in each group. The animal groups consisted of (1) saline control at 6 weeks and 9 months; (2) 2-times MPTP at 6 weeks; (3) 6-times MPTP at 6 weeks; (4) 2-times MPTP at 9 months; (5) 6-times MPTP at 9 months. Analysis was carried out to determine differences between all groups in the various anatomic regions. Because there was no significant difference between the saline groups at 6 weeks and 9 months for each anatomic region, these data were combined for analysis. Levels of dopamine and turnover ratio, defined as ([DOPAC + HVA]/dopamine) are shown in Table II and represented in Figure 3.

Dopamine levels in the dorsal putamen. Dopamine levels were significantly different in the dorsal putamen ($F = 23.2$, $df = 12$, $P < 0.001$). These data are represented in Figure 3A. Dopamine levels were significantly lower in the 2-time injected at 6 weeks (5.4 ± 5.2 ng dopamine/mg protein, 3.4% of control, $P < 0.001$), 6-time injected at 6 weeks (1.1 ± 0.3 ng dopamine/mg protein, 0.7% of saline control, $P < 0.001$), 2-time injected at 9 months (28.2 ± 11.1 ng dopamine/mg protein, 17.5% of saline control, $P < 0.001$), 6-time injected at 9 months (2.60 ± 0.3 ng dopamine/mg protein, 1.6% of saline control, $P < 0.001$) compared to saline (161.1 ± 18 mg ng dopamine/mg protein). There was no statistically significant difference between any MPTP-injected groups although there was a slight elevation of dopamine in the 2-time MPTP-injected group at 9 months that did not reach statistical significance.

Dopamine turnover ratio in the dorsal putamen. In the dorsal putamen, the dopamine turnover ratio was significantly different between groups ($F = 27.73$, $df = 12$, $P < 0.0001$). These data are represented in Figure 3B. The 2-time injected at 6 weeks (75.3 ± 37.7, $P < 0.0001$) and 6-time injected at 6 weeks (44.9 ± 6.7, $P < 0.01$) groups were significantly greater than saline control (1.2 ± 0.2). The turnover ratio in the 2-time MPTP-injected group was significantly higher than all other groups ($P < 0.01$).

Dopamine levels in the ventral putamen. Dopamine levels were different in the ventral putamen between groups ($F = 6.1$, $df = 12$, $P < 0.01$). These data are shown in Table I. These differences reached significance between the 2-time MPTP injected at 6 weeks (17.4 ± 16.9 ng dopamine/mg protein, 12.4% of saline control) compared to saline ($P < 0.05$) and the 6-time MPTP-injected group at 6 weeks (12.1 ± 4.5 ng dopamine/mg protein, 8.7% of saline control) compared to saline control ($P < 0.05$).

Dopamine turnover ratio in the ventral putamen. In the ventral putamen, the turnover ratio was different between groups ($F = 7.3$, $df = 12$, $P < 0.05$). These data are shown in Table I. This difference reached significance in the 2-time MPTP-injected group at 6 weeks (98.6 ± 62.5, $P < 0.05$) compared to saline control (1.6 ± 0.2) and all other groups.

Dopamine levels in the dorsal caudate nucleus. Dopamine levels were significantly different in the

TABLE II. HPLC Analysis of Dopamine and its Metabolites[†]

Treatment	Time point	Region	Dopamine (ng/mg protein)	DOPAC (ng/mg protein)	HVA (ng/mg protein)	Turnover ratio
Saline		Dorsal caudate (n = 6)	95.0 ± 6.3	11.7 ± 1.7	81.6 ± 10.5	1.0 ± 0.1
		Ventral caudate (n = 6)	133.6 ± 25.0	15.0 ± 1.2	121.7 ± 20.4	1.1 ± 0.1
		Dorsal putamen (n = 6)	161.1 ± 18.1	23.5 ± 4.0	168.2 ± 15.6	1.2 ± 0.2★★
		Ventral putamen (n = 6)	138.9 ± 25.4	22.0 ± 5.1	176.2 ± 13.9	1.6 ± 0.2★★
2 × MPTP	6 weeks	Dorsal caudate (n = 3)	6.6 ± 6.3★	1.7 ± 1.5	29.3 ± 17.6	40.0 ± 21.5★
		Ventral caudate (n = 3)	16.6 ± 16.1★	2.8 ± 2.2	29.3 ± 11.6	31.2 ± 18.6★
		Dorsal putamen (n = 3)	5.4 ± 5.2★	1.2 ± 1.1	41.5 ± 19.8★	75.3 ± 37.7★
		Ventral putamen (n = 3)	17.4 ± 16.9★	3.4 ± 2.9	63.2 ± 14	98.6 ± 62.5★
	9 months	Dorsal caudate (n = 3)	24.1 ± 6.4★	9.8 ± 3.5	61.6 ± 8.2	3.7 ± 1.5
		Ventral caudate (n = 3)	68.6 ± 18.7	15.0 ± 5.7	88.9 ± 7.5	1.7 ± 0.4
		Dorsal putamen (n = 3)	28.2 ± 11.1★	8.5 ± 3.8	102.8 ± 24.7	5.6 ± 2.3★★
		Ventral putamen (n = 3)	83.9 ± 32.2	14.3 ± 4.0	174.4 ± 31.2	2.8 ± 0.7★★
6 × MPTP	6 weeks	Dorsal caudate (n = 3)	0.6 ± 0.2★	0.5 ± 0.1	22.7 ± 3.5	52.3 ± 20.3★
		Ventral caudate (n = 3)	5.9 ± 2.8★	2.4 ± 0.4	36.9 ± 2.6	17.0 ± 11.9
		Dorsal putamen (n = 3)	1.1 ± 3★	0.9 ± 0.3	42.5 ± 11.8★	44.9 ± 6.7★·★★
		Ventral putamen (n = 3)	12.1 ± 4.5★	6.8 ± 2.9	98.9 ± 17.2	11.5 ± 3.9★★
	9 months	Dorsal caudate (n = 3)	8.0 ± 4.0★	2.6 ± 0.7	73.4 ± 29.5	20.9 ± 12
		Ventral caudate (n = 3)	31.5 ± 13.9	10.2 ± 2.3	89.2 ± 26.0	5.4 ± 2.5
		Dorsal putamen (n = 3)	2.6 ± 27★	2.12 ± .07	55.7 ± 10.3	23.2 ± 5.7★★
		Ventral putamen (n = 3)	35.8 ± 14.2	11.8 ± 0.82	71.3 ± 25.6	4.8 ± 1.8★★

[†]Specific anatomical regions (dorsal caudate, ventral caudate, dorsal putamen, or ventral putamen) from individual groups were analyzed using a one-way ANOVA with a Bonferroni post-hoc analysis.
★Significantly different from the saline group ($P < 0.05$).
★★Significantly different from the 2 × MPTP/6 weeks group ($P < 0.05$).

dorsal caudate nucleus ($F = 66.5$, $df = 12$, $P < 0.001$). These data are represented in Figure 3C. Dopamine levels were significantly lower in the 2-time injected at 6 weeks (6.6 ± 6.3 ng dopamine/mg protein, 6.9% of control, $P < 0.001$), 6-time injected at 6 weeks (0.6 ± 0.2 ng dopamine/mg protein, 0.6% of saline control, $P < 0.001$), 2-time injected at 9 months (24.1 ± 6.4 ng dopamine/mg protein, 25.4% of saline control, $P < 0.001$), 6-time injected at 9 months (8.0 ± 4.0 ng dopamine/mg protein, 8.4% of saline control, $P < 0.001$) compared to saline (95.0 ± 6.3 mg ng dopamine/mg protein). There was no statistically significant difference between any MPTP-injected groups although there was a slight elevation of dopamine in the 2-time MPTP-injected group at 9 months, which did not reach statistical significance.

Dopamine turnover ratio in the dorsal caudate nucleus. In the dorsal caudate nucleus, the dopamine turnover ratio was significantly different between groups ($F = 5.8$, $df = 12$, $P < 0.05$). These data are represented in Figure 3D. The 2-time injected at 6 weeks (40.0 ± 21.5, $P < 0.05$) and 6-time injected at 6 weeks (52.3 ± 20.3, $P < 0.05$) groups were significantly greater than saline control (1.0 ± 0.1).

Dopamine levels in the ventral caudate nucleus. Dopamine levels were different in the ventral caudate nucleus between groups ($F = 6.1$, $df = 12$, $P < 0.02$). These data are shown in Table I. These differences reached significance between the 2-time MPTP injected at 6 weeks (16.6 ± 16.1 ng dopamine/mg protein, 12.4% of saline control, $P < 0.05$) and the 6-time

MPTP injected at 6 weeks (5.9 ± 2.8 ng dopamine/mg protein, 4.4% of saline control, $P < 0.05$) compared to saline (133.6 ± 25.0 ng dopamine/mg protein).

Dopamine turnover ratio in the ventral caudate nucleus. In the ventral caudate, the dopamine turnover ratio was significantly different between groups ($F = 4.7$, $df = 12$, $P < 0.05$). These data are shown in Table I. The 2-time injected at 6 weeks (31.2 ± 18.6, $P < 0.05$) was significantly different from saline control (1.1 ± 0.1).

Analysis of TH Protein Expression

The pattern of expression of TH protein in the caudate nucleus and putamen of animals from all groups was determined using both immunocytochemistry and western immunoblotting (Fig. 4). In saline injected animals, TH-immunoreactivity (TH-ir) at the level of the mid-striatum showed intense staining throughout the entire caudate nucleus and putamen as seen in coronal sections at low magnification (Fig. 4A). At high magnification the staining appeared as a dense fibrous network with staining in both puncta and dark fibers of various thicknesses (Fig. 4B). At 6 weeks after the last MPTP injection, tissues from the 2-time and 6-time injected groups showed severe depletion of TH-ir. Remaining TH-ir appeared as light background staining with dark staining TH-ir fibers. The TH-ir fibers were most apparent in the 2-time compared to the 6-time injected groups (compare Fig. 4D and 4H). Examination of TH-ir at 9 months after the last injection of MPTP showed a
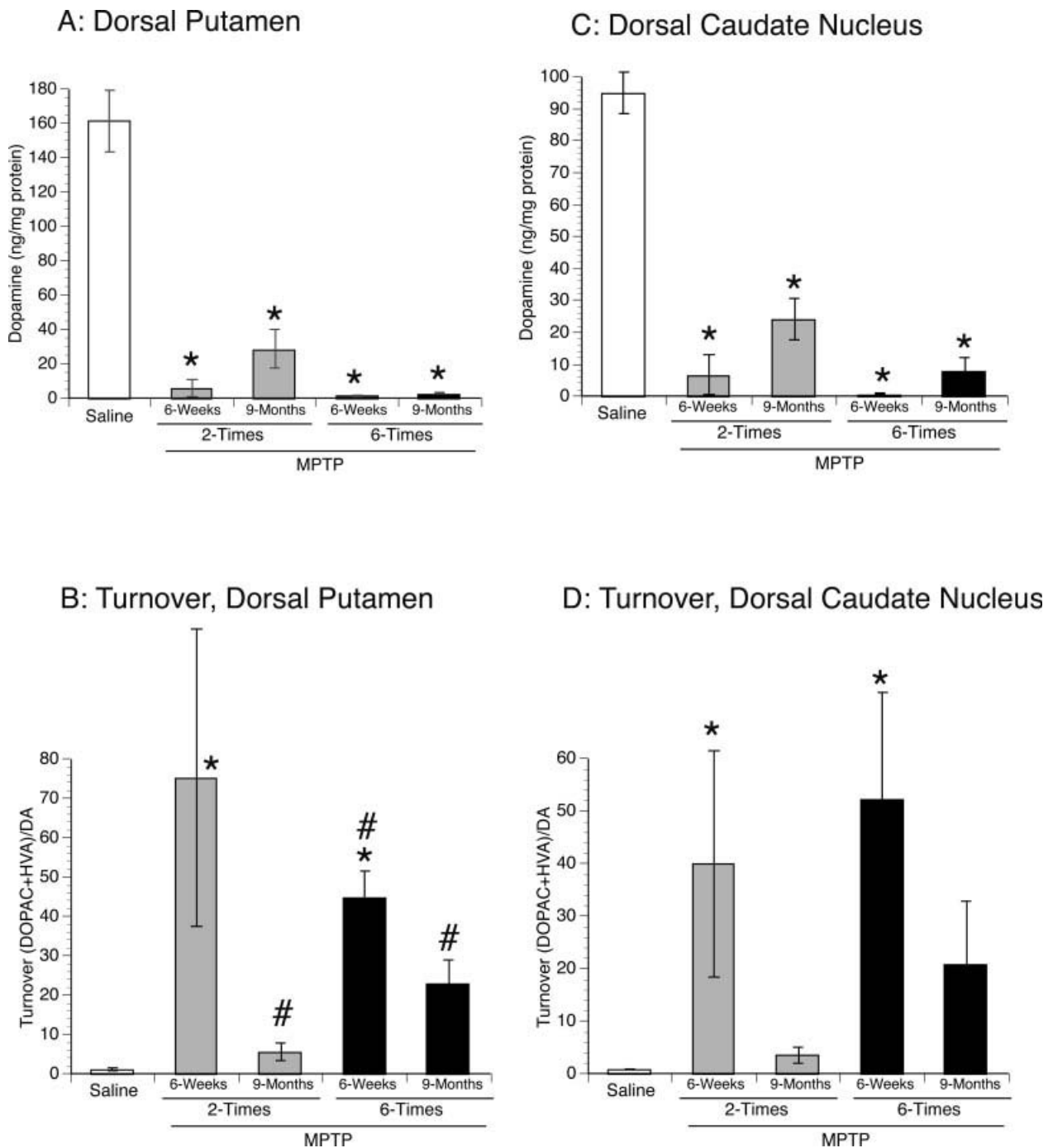
A: Dorsal Putamen

C: Dorsal Caudate Nucleus

B: Turnover, Dorsal Putamen

D: Turnover, Dorsal Caudate Nucleus

Fig. 3. Neurochemical analysis of dopamine and its metabolites. Data are shown for the analysis of dopamine from (**A**) the dorsal putamen and (**C**) the dorsal caudate nucleus from at least three animals per group except saline where $N = 6$. Saline groups consist of pooled data from tissues collected at both the 6-week and 9-month time points because statistical analysis showed no difference. Tissue for analysis was collected from the 2-time and 6-time MPTP-injected groups at either 6 weeks or 9 months after the last injection of MPTP. Dopamine turnover defined as (DOPAC + HVA)/DA, is depicted in (**B**) for the dorsal putamen and (**D**) the dorsal caudate nucleus. *Indicates statistical significance compared to saline ($P < 0.05$). #Indicates statistical significance compared to the 2-time MPTP-injected group at 6 weeks ($P < 0.05$).
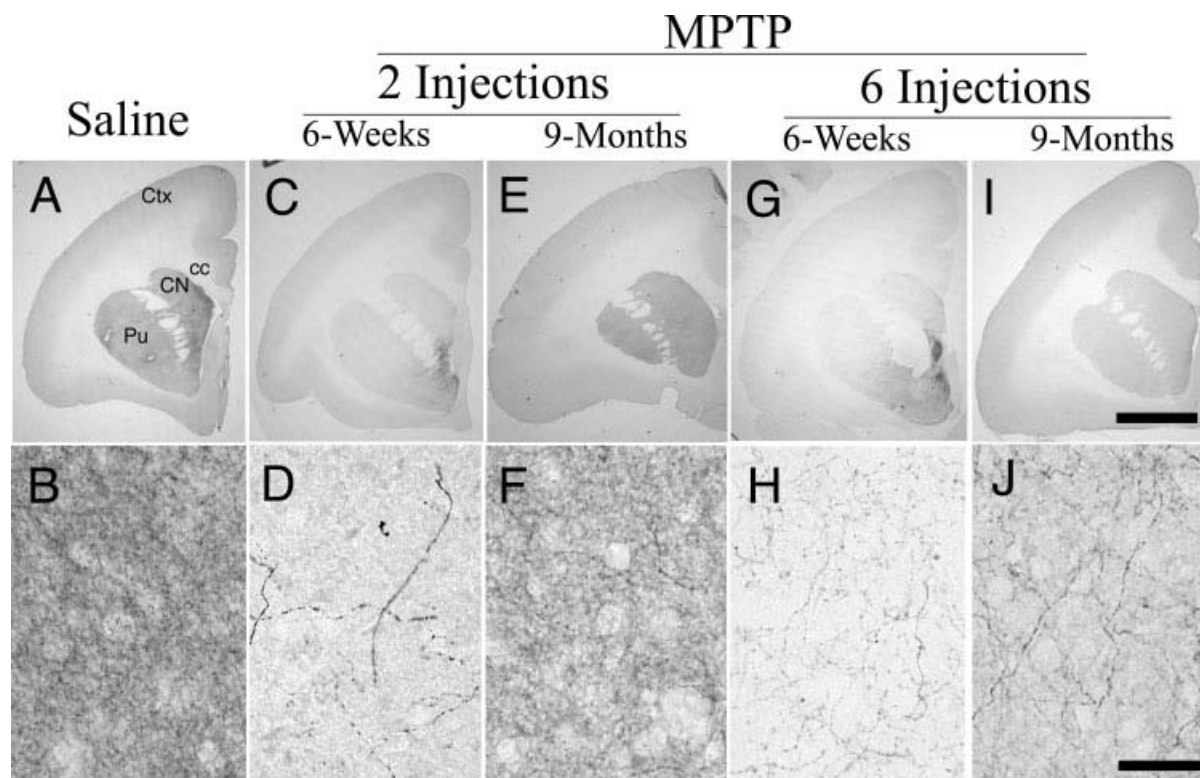
Fig. 4. Immunohistochemical staining for TH protein in the caudate nucleus and putamen. **Upper panels:** Representative images at low magnification of hemi-sections in the coronal plane at the level of the mid-striatum at the anterior–posterior plane between 14–15 mm. **Lower panels:** High magnification images obtained from the most dorsal quadrant of the caudate nucleus. **A:** TH-ir is seen as dark immuno-staining throughout the caudate nucleus and putamen. **B:** At higher magnification dark TH-ir appears as a thick fibrous network. **C:** At 6-weeks after two injections of MPTP there is a significant reduction in TH-ir throughout the caudate nucleus and putamen and (**D**) only a light fibrous TH-ir network remains. **E:** At 9 months after the last of the 2-time MPTP injections TH-ir increases throughout the striatum and (**F**) an increase in the fibrous network is observed. **G:** At 6 weeks after the last injection in the 6-time MPTP regimen results in a severe depletion of TH-ir (**H**), which appears as very light staining with a small number of distinct fibers remaining. **I:** At 9 months after the last of the series of six injections of MPTP there is an increase in the degree of TH-ir (**J**) corresponding to an increase in the degree of TH-ir fiber density. CN, caudate nucleus; Pu, putamen; cc, corpus callosum; ctx, cortex. Scale bar = 0.5 mm (I, images in the top row); 15 µm (J, images in the bottom row).

partial return of TH-ir in both the 2-time (compare Fig. 4C and 4E) and 6-time injected groups (compare Fig. 4G and 4I). The degree of TH-ir return was greater in the 2-time injected group (compare Fig. 4E and 4I) with the 2-time injected group showing a greater intensity of TH-ir (compare Fig. 4F and 4J), in the amount of fibers and neuropil background.

Western immunoblotting analysis was carried out to determine the relative degree of TH protein expression in the dorsal putamen from animals in all groups. A representative immunoblot is shown in Figure 5. There was no difference in the level of TH protein in saline animals harvested at 6 weeks or 9 months. Therefore, the results from the saline animals were pooled. The relative amount of TH protein was significantly lower compared to saline control in the 2-time injected at 6 weeks ($16.2 \pm 0.6\%$ of saline control, $P < 0.01$), the 2-time injected at 9 months ($31.8 \pm 1.7\%$ of saline control, $P < 0.01$), 6-time injected at 6 weeks ($8.9 \pm 1.1\%$ of saline control, $P < 0.01$), and the 6-time injected at 9 months ($20.7 \pm 1.7\%$ of saline control, $P < 0.01$). The relative amount of TH protein in the 2-time MPTP injected at 9-months and the 6-time MPTP injected at 9 months showed a slight increase compared to the other MPTP-injected groups. This increase, however, did not reach significance.

Analysis of DAT Protein Expression

The pattern of expression of DAT protein in the caudate nucleus and putamen from animals in all groups was determined using immunohistochemistry (Fig. 6). In saline injected animals, DAT-immunoreactivity (DAT-ir) in coronal sections at the level of the mid-striatum showed intense staining throughout as shown at low magnification (Fig. 6A). At high magnification the staining appeared as a dense fibrous network similar to that seen with TH-ir with staining in both puncta and dark fibers of various thicknesses (Fig. 6B). Tissues from
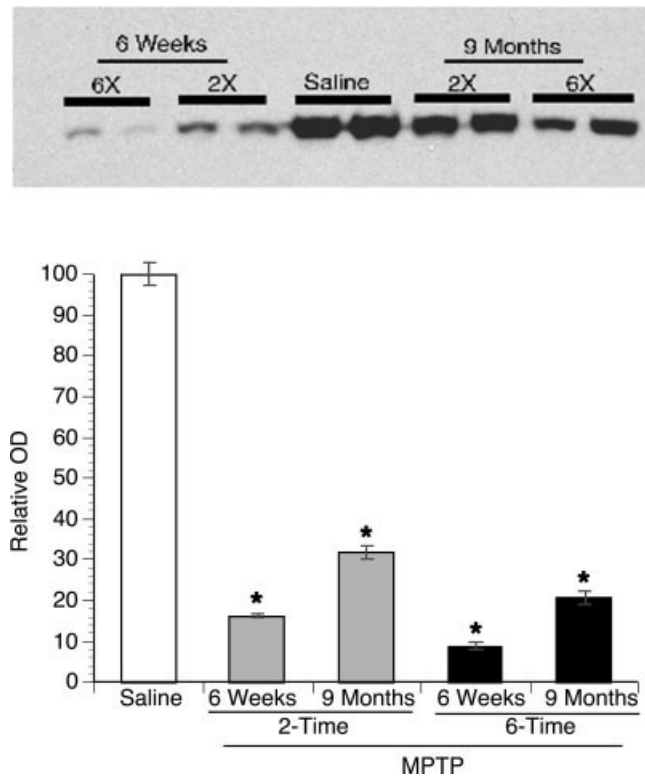
Fig. 5. Western immunoblot for TH protein in the caudate nucleus. **Upper panel:** Representative immunoblot comparing the degree of TH protein expression in the caudate nucleus from tissues collected from the 2-time and 6-time MPTP-injected groups at 6 weeks and 9 months after the last injection. Note the highest amount of expression in the saline group, depletion at 6 weeks in both MPTP-lesioned groups and partial return in both MPTP-lesion groups at 9 months. **Lower panel:** Relative optical density (OD) of immunoblot data derived from two samples from three different animals from each group. *MPTP-lesioned groups statistically significant from saline ($P < 0.05$).

both the 2-time and 6-time MPTP injection groups at 6 weeks post-injection of MPTP showed significant depletion of DAT-ir. Remaining DAT-ir appeared as a light background staining with dark staining fibers. The degree of DAT-ir was greater in the 2-time injection group compared to the 6-time injected group (compare Fig. 6D and 6H). At 9 months after the last injection of MPTP, analysis of DAT-ir showed a partial return in both the 2-time injected (compare Fig. 6C and 6E) and 6-time injected groups (compare Fig. 6G and 6I). The degree of DAT-ir return was greater in the 2-time injected group (compare Fig. 6E and 6I) with the 2-time injected group showing a greater intensity of DAT-ir (compare Fig. 6F and 6J).

Western immunoblotting analysis was carried out to determine the relative degree of DAT protein expression in the dorsal putamen from animals in all groups. There was no difference in the level of DAT protein in saline animals harvested at 6 weeks or 9 months. There-fore, the results from the saline animals were pooled. The relative amount of DAT protein was significantly lower compared to saline control in the 2-time injected at 6 weeks ($13.0 \pm 1.3\%$ of saline control, $P < 0.05$), 2-time injected at 9 months ($43.1 \pm 2.8\%$ of saline control. $P < 0.05$), 6-time injected at 6 weeks ($15.9 \pm 3.2\%$ of saline control, $P < 0.05$), and 6-time injected at 9 months ($32.6 \pm 5.0\%$ of saline control, $P < 0.05$). DAT expression was greater significantly, however, in the 2-time injected at 9 months and the 6-time injected at 9 months compared to the MPTP-injected groups at 6 weeks ($P < 0.05$).

Analysis of DARPP-32 Protein Expression

The pattern of expression of DARPP-32 protein in coronal sections of the mid–caudate nucleus and putamen of animals from all groups was determined using immunocytochemistry (Fig. 7). The highest degree of immunoreactivity was seen in cell bodies throughout the striatum, with some neuropil staining. After MPTP administration, the 2-time injected at 6 weeks and the 6-time injected at 6 weeks showed reduction in the intensity of cell body and neuropil staining. The degree of immunoreactivity returned to levels that were similar to that of the saline control groups in the 2-time injected at 9 months and 6-time injected at 9 months.

The relative optical density of DARPP-32 protein immunoreactivity was carried out in the dorsal region of these sections from all groups (Fig. 7). Optical density is reported as a % relative to saline. There was no difference in the optical density of DARPP-32 protein in saline animals harvested at 6 weeks or 9 months. Therefore, the results from the saline animals were pooled. The optical density of DARPP-32 protein was significantly lower in the 2-time injected at 6 weeks ($76.4 \pm 3.7\%$ of saline) and the 6-time injected at 6 weeks ($63.2 \pm 2.5\%$ of saline) compared to saline ($P < 0.001$). However the 2-time injected at 9 months and the 6-time injected at 9 months were not significantly different from the saline group ($101.1 \pm 1.9\%$ and $91.9 \pm 3.7\%$, respectively).

DISCUSSION

The systemic administration of the neurotoxicant MPTP in the nonhuman primate squirrel monkey (*Saimiri sciureus*) leads to the selective destruction of dopaminergic neurons in the substantia nigra pars compacta and ventral tegmental area and the depletion of dopamine in the caudate nucleus and putamen (Burns et al., 1983; Langston et al., 1984). In addition to the neurochemical changes, MPTP-lesioned nonhuman primates display profound deficits in motor behavior that resemble features of parkinsonism including akinesia, bradykinesia (slowness), postural instability (balance), and freezing (Petzinger and Langston, 1998). Similar to patients with idiopathic PD, MPTP-lesioned animals respond to dopamine replacement therapy (levodopa [L-DOPA] + carbidopa) with improvement of motor features and also display L-
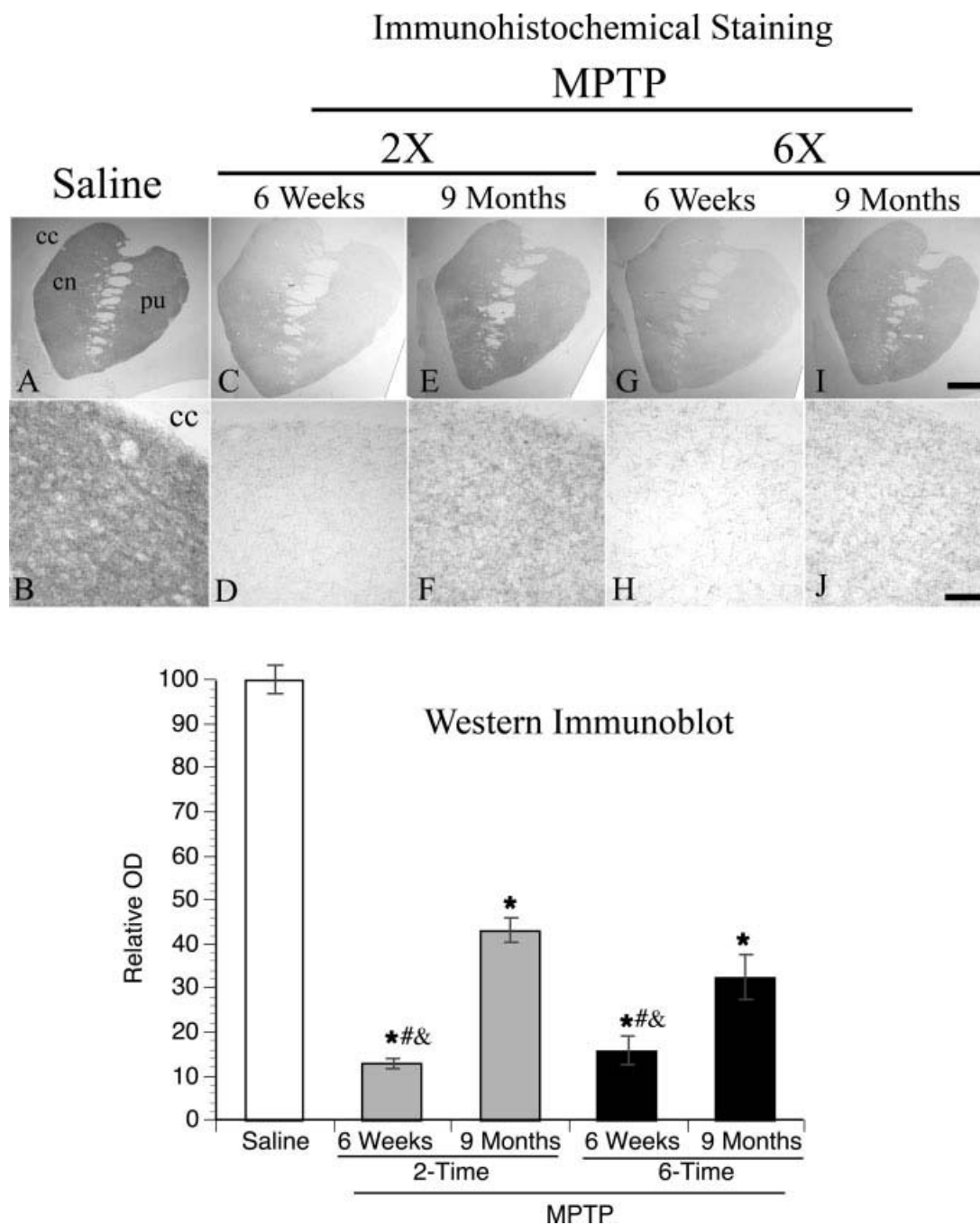
Immunohistochemical Staining

Fig. 6. Immunohistochemical staining for dopamine transporter in the caudate nucleus and putamen. **Upper panel:** Series of photomicrographs images shows coronal sections through the mid-striatum at level in the anterior–posterior plane at 14–16 mm were stained for DAT-ir and representative sections photographed at low magnification (12.5× in upper panels; scale bar = 0.2 mm) and at high magnification (400× in lower panels; scale bar = 6 μm). **A:** Sections from saline injected animals showed intense dark staining throughout the caudate nucleus and putamen and (**B**) appeared as a dark fibrous network with a high background. **C:** At 6 weeks after the last injection in the 2-time MPTP regimen DAT-ir was significantly reduced with (**D**) a small number of dark DAT-ir fibers remaining on a lightly immunoreactive background. **E:** After 9 months the 2-time MPTP injection regimen showed the return of DAT-ir that (**F**) appeared as a fibrous network of various sizes with increased DAT-ir background. **G:** At 6 weeks after the regimen of six injections of MPTP DAT-ir was reduced significantly throughout the caudate nucleus and putamen with (**H**) a small number of DAT-ir fibers remaining. **I:** At 9 months after the regimen of six injections of MPTP there was a slight return of DAT-ir that (**J**) appeared as an increase in the staining of fibers of various sizes resulting in increased background staining. **Lower panel:** Relative optical density for Western immunoblot analysis of DAT expression in the dorsal putamen. *Indicates statistical significance compared to the saline control group ($P < 0.05$). #Indicates statistical significance compared to the 2-time MPTP-injected group at the 9-month time point ($P < 0.05$). &Indicates statistical significance compared to the 6-time MPTP-injected group at the 9-month time point ($P < 0.05$).
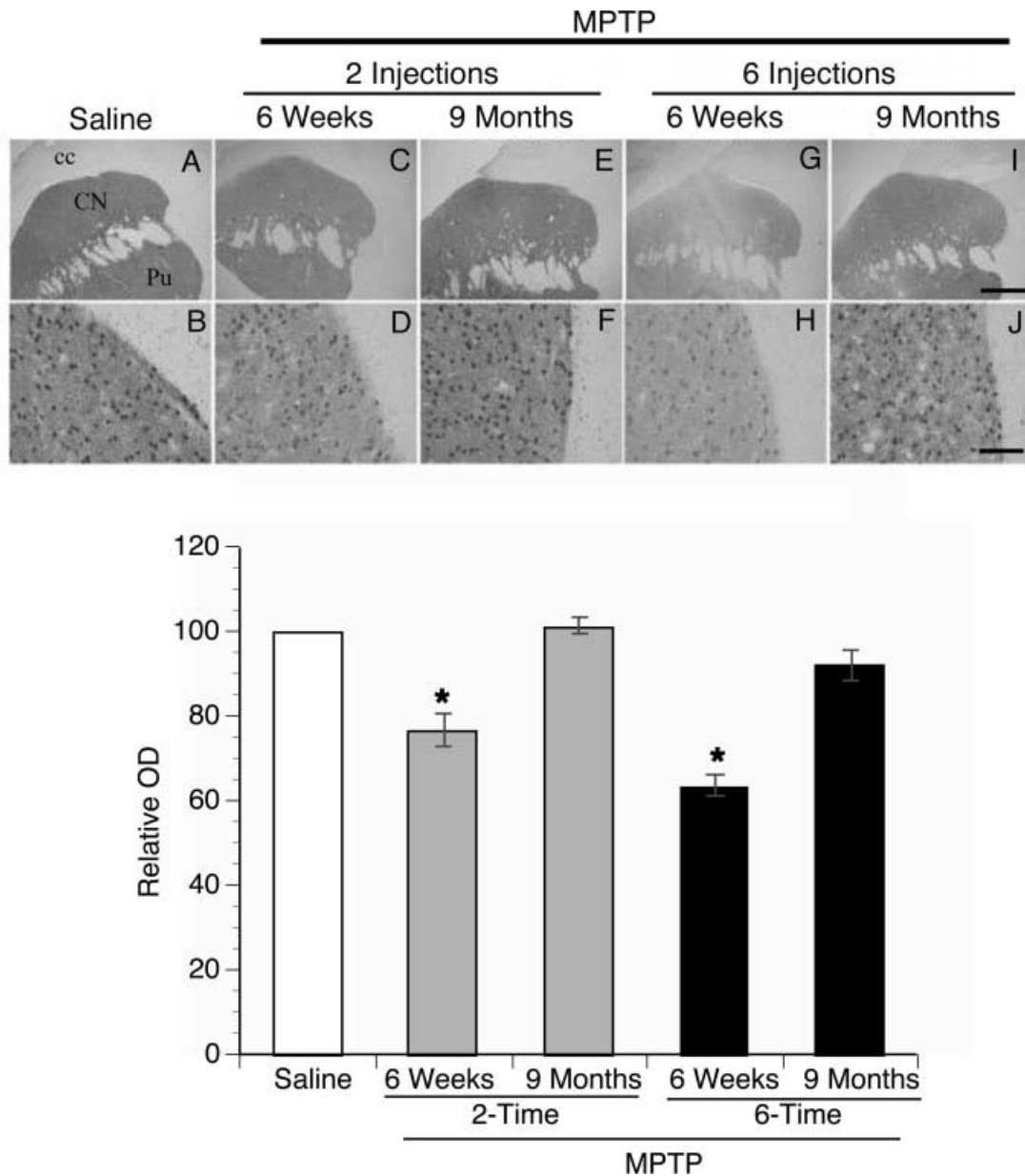
Fig. 7. Immunohistochemical staining for DARPP-32 protein in the caudate nucleus and putamen. **Upper panel:** Series of photomicrographs images shows coronal sections through the mid-striatum were stained for DARPP-32 immunoreactivity and representative sections photographed at low magnification (12.5× in the upper panels; scale bar = 0.25 mm) and at high magnification in a region representing the dorsal putamen adjacent to the corpus callosum (400× in the lower panels; scale bar = 7 μm). **A:** Saline animals showed immunoreactivity throughout the striatum at low magnification, (**B**) which appeared as staining predominantly within cell nuclei and to a lesser extent in the neuropil. At 6 weeks after MPTP-lesioning the 2-time and 6-time injected animals showed a reduction in the intensity of overall immunoreactivity (**C,G**), which appeared as reduced staining within both cell bodies themselves and to a lesser degree the neuropil (**D,H**). Immunostaining was less intense in the 6-time injected group compared to the 2-time injected group (D,H). At 9 months after MPTP-lesioning immuno-staining in the 2-time injection group was similar to that seen in saline animals (**E,F**). Immuno-staining at 9 months after MPTP-lesioning in the 6-time injection group had immuno-staining (I and J) that was less intense then both the saline and 2-time injected animals at the same time point. **Lower panel:** Relative optical density of DARPP-32 immuno-staining in the dorsal putamen in all treatment groups ($n = 8$ sections/group). *Indicates statistical significance compared to the saline control group ($P < 0.05$). CN, caudate nucleus; Pu, putamen; cc, corpus callosum.

DOPA–related motor complications, including dyskinesia (Schneider, 1989; Boyce et al., 1990a,b; Petzinger et al., 2001).

In our study, as in others, the degree of motor behavioral deficits is dependent on the severity of MPTP lesioning because monkeys administered two injections of MPTP (2.0 mg/kg, free-base per injection) have mild symptoms whereas animals receiving a series of six injections of MPTP display more severe parkinsonism. Additionally, we observed that motor recovery was apparent by 6 weeks post-MPTP and was complete in mild and severely lesioned animals by 9 months, with animals appearing indistinguishable from normal non-lesioned animals. Fully recovered animals display normal spontaneous movements (jumping and climbing throughout cage), as well as normal hand dexterity and balance. Behavioral recovery has been reported in several species of nonhuman primates after MPTP-lesioning (Rose et al., 1989a,b; Elsworth et al., 1990; Kurlan et al., 1991; Elsworth et al., 2000). Interestingly, the reported degree of recovery is variable and may be dependent on several factors including the time after lesioning, the species, and the mode of behavioral assessment. For example, studies in the MPTP-lesioned marmoset showed recovery with respect to gross akinesia by 3–4 months post-lesioning but "lack of spontaneous movement and poor coordination of activities" were still evident and persisted at 18 months post-MPTP-lesioning (Rose et al., 1989a,b; Ueki et al., 1989).

Using our MPTP lesioning regimen, we saw significant dopamine depletion in the caudate nucleus and putamen at 6 weeks post-lesioning that persisted at 9 months despite motor behavioral recovery. This degree of depletion, however, did show regional differences (dorsal versus ventral caudate nucleus and putamen). For example, at 6 weeks after the last injection of MPTP dopamine levels in the dorsal putamen and dorsal caudate nucleus were depleted >93% compared to dopamine levels in saline control. At 9 months after the last injection of MPTP, when full motor recovery was observed, dopamine levels in the dorsal putamen and dorsal caudate nucleus remained low. This was in contrast to both the ventral putamen and ventral caudate nucleus where the degree of depletion was less than that seen in the respective dorsal regions at 6 weeks and the return of dopamine at 9 months after MPTP-lesioning was greater. Our findings are similar to other nonhuman primate studies that have reported reversal of parkinsonian features despite persistent dopamine loss (Rose et al., 1989a; Schneider and Rothblat, 1991; Gnanalingham et al., 1995; Rothblat and Schneider 1995). These studies also reported greater return of dopamine in the ventral striatum, the greatest extent measured in the nucleus accumbens, the most ventral aspect of the striatum (Rose et al., 1989b; Gnanalingham et al., 1995). Analogous to the MPTP-lesioned nonhuman primate, studies reporting behavioral recovery despite extensive dopamine depletion have been made in the MPTP-lesioned cat model examining the reversal of akinesia, bradykinesia, and sensorimotor neglect and in the 6-OHDA-lsioned rat examining the reversal of sensorimotor neglect, aphagia, adipsia, and bradykinesia (Ungerstedt, 1971; Zigmond and Stricker, 1973; Marshall and Teitelbaum, 1974; Marshall and Gotthelf, 1979; Schneider et al., 1994).

Pre- and post-synaptic mechanisms may play a role in compensating for the incomplete return of dopamine, and may underlie behavioral recovery after neurotoxicant injury to the basal ganglia. The pre-synaptic mechanisms include increased dopamine biosynthesis due to altered affinity of TH for its cofactors, increased expression of TH protein, increased dopamine release, and decreased synaptic dopamine uptake. These pre-synaptic adaptations in remaining dopaminergic neurons are thought to lead to normalization of extracellular levels of dopamine especially in animals with lesions resulting in <80% depletion of total striatal intracellular and extracellular dopamine pools (Robinson and Wishaw, 1988; Zhang et al., 1988; Altar and Marien 1989; Abercrombie et al., 1990; Castaneda et al., 1990). Animals with >80% total dopamine depletion show only partial normalization of extracellular striatal dopamine (Castaneda et al., 1990). The relative contribution of post-synaptic changes in targets of dopaminergic neurons to behavioral recovery is thought to be dependent on the extent of dopamine depletion within the extracellular space (Zigmond et al., 1984, 1990; Zhang et al., 1988). These post-synaptic changes include altered dopamine receptor number and function and their influence on the expression of neuropeptides, second messengers, transcription factors, and down-stream effector molecules, especially within striatal medium–spiny neurons (Creese et al., 1977; Zigmond et al., 1984).

In the MPTP-lesioned squirrel monkey, we observed an increase in dopamine metabolites relative to dopamine, defined as the turnover ratio ([DOPAC + HVA]/dopamine), during the early motor behavioral recovery period (at 6 weeks). Turnover ratio showed regional differences within the striatum and was highest in the dorsal putamen and dorsal caudate nucleus. Consistent with our study, others have reported elevated turnover ratio highest in those areas of the striatum (dorsal region) with the greatest amount of dopamine depletion (Onn et al., 1986; Schneider and Rothblat, 1991; Elsworth et al., 2000). At 9 months after MPTP-lesioning when motor behavioral recovery was complete, however, turnover ratio was diminished in all regions of the striatum compared to the 6-week time point. This observation of a decline in turnover ratio during motor recovery has also been observed in the MPTP marmoset (Gnanalingham et al., 1995). Changes in turnover ratio is thought to be due to changes in the rate and firing of dopaminergic neurons or increase in dopamine release from surviving dopaminergic neurons (Hollerman and Grace, 1990). The result of elevated turnover ratio is to attempt to maintain extracellular levels of dopamine.

The level of extracellular dopamine is also influenced by the DAT, a protein localized to nigrostriatal terminals (Gainetdinov et al., 2002). Decreased DAT

expression that was observed after MPTP-lesioning may lead to increased synaptic levels of dopamine and increase in the diffusion of dopamine to depleted regions (Schneider et al., 1994; Rothblat and Schneider, 1999) during the early period of motor behavioral recovery in the squirrel monkey. At 9 months, when motor behavioral recovery was complete, DAT expression increased throughout the striatum, even in the dorsal striatum where dopamine levels remained persistently low. An increased DAT expression is likely due to the presence of sprouting in surviving nigrostriatal dopaminergic neurons (Finkelstein et al., 2000; Jakowec et al., 2004). Similar to striatal dopamine and its metabolites, there were regional differences in DAT expression, with the greatest return noted in the ventral striatum (data not shown). The return of striatal DAT during motor recovery seems to vary among studies and may depend on the degree and method of lesioning, animal species, neurotoxicant (MPTP vs. 6-OHDA), and the time point of analysis. Most studies suggest that sprouting may rely on some degree of surviving dopaminergic neurons to serve as a template, because partial lesioning shows more robust expression of markers for sprouting in comparison to near-complete lesioning (Frohna et al., 1997; Mitsumoto et al., 1998; Finkelstein et al., 2000).

Tyrosine hydroxylase is an enzyme important in the biosynthesis of dopamine and is often considered a marker of nigrostriatal neuron sprouting (Bezard et al., 2000; Rothblat et al., 2001; Jakowec et al., 2004). At 6 weeks post-MPTP lesioning, when animals are displaying early motor behavioral recovery, dopamine levels are low and TH–immunoreactivity is severely depleted. At 9 months when animals are recovered fully, however, TH expression shows partial return. This alteration in TH is analogous to DAT, and may also be an indicator of neuronal sprouting. Interestingly, increased TH expression was observed within the dorsal putamen, despite persistently low dopamine levels (up to 98% depletion). Similar to the conclusions of others, our study suggests that sprouting of TH immunoreactive fibers may precede the biosynthesis and return of significant levels of striatal dopamine (Jakowec et al., 2004; Mitsumoto et al., 1998).

One post-synaptic marker of the functional integrity of striatal medium spiny neurons is DARPP-32. DARPP-32 is a down-stream effector molecule that regulates a number of enzymatic and receptor mediated cellular processes that are essential for normal basal ganglia function (Ouimet et al., 1984; Anderson and Reiner, 1991; Nishi et al., 1997). The activity of DARPP-32 can be regulated by its phosphorylated state as well as expression levels, which may be influenced by dopaminergic or glutamatergic neurotransmission (Greengard et al., 1999; Nishi et al., 2005). Most studies on DARPP-32 expression have examined its phosphorylation state shortly after injury and not total protein levels (Raisman-Vozari et al., 1990). In our study, the relative DARPP-32 protein levels, as indicated by a pan-specific antibody, were measured by optical density of striatal immunostained tissues. DARPP-32 immunostaining was initially reduced in cells during the early period of behavioral recovery (6 weeks post–MPTP) in the 2-time and 6-time MPTP-injected groups. However, by 9 months and after motor behavioral recovery, DARPP-32 immunostaining had fully returned despite regional areas of persistent and marked dopamine depletion. Given the dichotomy between the return of DARPP-32 protein expression and continued dopamine depletion suggests that other non–dopaminergic systems, such as the glutamatergic, may make a more significant contribution to the function of medium spiny neurons during motor behavioral recovery in the MPTP-lesioned squirrel monkey (Greengard et al., 1999; Wang et al., 2004; Nishi et al., 2005). We are currently investigating this hypothesis in our lab through the administration of specific glutamate receptor antagonists.

In conclusion, our study indicates that several months after MPTP-lesioning, squirrel monkeys display full motor behavioral recovery using a clinical rating scale that measures parkinsonian features. Interestingly, the return of striatal dopamine appears incomplete with more pronounced return in the ventral striatum in comparison to the dorsal striatum. This finding is consistent with other reports using neurotoxicant-lesioning models of the basal ganglia in both rodents and other species of nonhuman primates. Elevated dopamine turnover ratio and decreased DAT expression seem to be two pre-synaptic mechanisms that may be important for the compensation of dopamine depletion in early behavioral recovery. The increase of both TH and DAT expression during the time when motor behavioral recovery is complete suggests neuronal sprouting as an important mechanism in late stage recovery even within dopamine depleted regions. In addition, post-synaptic mechanisms within the medium spiny neurons, as indicated by altered DARPP-32 expression, seem to be potentially important targets for investigation.

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Exercise-Induced Behavioral Recovery and Neuroplasticity in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Lesioned Mouse Basal Ganglia

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Physical activity has been shown to be neuroprotective in lesions affecting the basal ganglia. Using a treadmill exercise paradigm, we investigated the effect of exercise on neurorestoration. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse model provides a means to investigate the effect of exercise on neurorestoration because 30–40% of nigrostriatal dopaminergic neurons survive MPTP lesioning and may provide a template for neurorestoration to occur. MPTP-lesioned C57 BL/6J mice were administered MPTP (four injections of 20 mg/kg free-base, 2 hr apart) or saline and divided into the following groups: (1) saline; (2) saline + exercise; (3) MPTP; and (4) MPTP + exercise. Mice in exercise groups were run on a motorized treadmill for 30 days starting 4 days after MPTP lesioning (a period after which MPTP-induced cell death is complete). Initially, MPTP-lesioned + exercise mice ran at slower speeds for a shorter amount of time compared to saline + exercise mice. Both velocity and endurance improved in the MPTP + exercise group to near normal levels over the 30-day exercise period. The expression of proteins and genes involved in basal ganglia function including the dopamine transporter (DAT), tyrosine hydroxylase (TH), and the dopamine D1 and D2 receptors, as well as alterations on glutamate immunolabeling were determined. Exercise resulted in a significant downregulation of striatal DAT in the MPTP + exercise compared to MPTP nonexercised mice and to a lesser extent in the saline + exercised mice compared to their no-exercise counterparts. There was no significant difference in TH protein levels between MPTP and MPTP + exercise groups at the end of the study. The expression of striatal dopamine D1 and D2 receptor mRNA transcript was suppressed in the saline + exercise group; however, dopamine D2 transcript expression was increased in the MPTP + exercise mice. Immunoelectron microscopy indicated that treadmill exercise reversed the lesioned-induced increase in nerve terminal glutamate immunola-
beling seen after MPTP administration. Our data demonstrates that exercise promotes behavioral recovery in the injured brain by modulating genes and proteins important to basal ganglia function.
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It has been established that neuroplasticity, as characterized by neurogenesis, synaptogenesis, and molecular adaptations, exists in the human nervous system. Animal models of brain injury have provided a means to both investigate and manipulate neuroplasticity. A heightened area of interest is the role that exercise plays in facilitating neuroplasticity in either the noninjured or injured brain (Fisher et al., 2001). Studies employing a variety of animal models of injury have shown that exercise can promote neuroplasticity and behavioral recovery in the hippocampus, cortex, and spinal cord (Kempermann et al., 2000). In

rodent models of basal ganglia injury, exercise has been shown to be neuroprotective (Tillerson et al., 2001, 2003; Tillerson and Miller, 2002). By restraining the unimpaired limb immediately after injury and forcing use of the impaired upper limb, behavioral and neurochemical sparing were demonstrated in the 6-hydroxydopamine (6-OHDA)-lesioned rat. This suggested that forced use of the impaired limb protected dopaminergic cells from the neurotoxic effects of 6-OHDA. In addition, Tillerson et al. (2003) reported behavioral improvement after treadmill exercise in two rodent models of basal ganglia injury (the 6-OHDA rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]-lesioned mouse) (Tillerson et al., 2003). Immediate exposure to treadmill training within 12 hr of injury was associated with attenuation of dopamine loss. The investigators concluded that exercise might work largely through neuroprotective mechanisms because exercise was started within 12 hr of lesioning, and MPTP and 6-OHDA may take several days to complete cell death (Sauer and Oertel, 1994; Jackson-Lewis et al., 1995). The implication of these neuroprotective studies is that exercise may be helpful in delaying or preventing Parkinson's disease in healthy individuals (Sasco et al., 1992).

The interest in our laboratory is to investigate the role of exercise in promoting repair of the injured basal ganglia. We define this as neurorestoration, which is the capacity of surviving dopaminergic neurons to adapt after injury with potential behavioral benefits. The MPTP-lesioned mouse model of basal ganglia injury provides a means to investigate neurorestoration because 30–40% of the substantia nigra pars compacta (SNpc) dopaminergic neurons survive the lesioning regimen. Despite a 90% loss of striatal dopamine, these mice display robust and reproducible return of striatal dopamine, tyrosine hydroxylase (TH) protein, and dopamine transporter (DAT) protein 2–3 months after MPTP lesioning. This suggests that surviving nigrostriatal dopaminergic neurons provide a template for neurorestoration and would therefore provide a means to investigate the effect of exercise on facilitating neurorestoration. In addition, MPTP-lesioned mice manifest motor behavioral deficits that can be monitored throughout the recovery process (Tillerson et al., 2003). The purpose of our study was to investigate the effect of treadmill exercise on neurorestoration using the MPTP-lesioned mouse model of basal ganglia injury by introducing exercise 4 days after MPTP lesioning, a time point well after cell death is complete. Because TH, DAT, dopamine (D1 and D2) receptors and glutamate storage have been shown to be altered in the MPTP-lesioned mouse model (Jakowec et al., 2004), we chose to examine these same parameters in our MPTP exercise paradigm. Exercise was continued for 30 days to parallel the 30-day recovery period when TH is beginning to return.

MATERIALS AND METHODS

Housing and Acclimation of Mice

Young adult (8–10 weeks old) male C57BL/6J mice supplied from Jackson Laboratory (Bar Harbor, ME) were used for this study. There were four treatment groups: (1) saline injected; (2) saline + exercise; (3) MPTP lesioned; and (4) MPTP lesioned + exercise. Three cohorts of mice, consisting of four groups of 10 mice/group were used (total $n + 120$ mice). Animals were housed six to a cage and acclimated to a 12-hr shift in light/dark cycle so that exercise occurred during the animals' normal wake period.

MPTP Lesioning

MPTP (Sigma, St. Louis, MO) was administered in a series of four intraperitoneal injections of 20 mg/kg (free-base) at 2-hr intervals for a total administration of 80 mg/kg. This regimen leads to a 60–70% loss of nigrostriatal neurons (as determined by unbiased stereologic techniques for both TH staining and Nissl substance in our laboratory) and an 80–90% depletion of striatal dopamine levels (Jackson-Lewis et al., 1995). Using this regimen, nigrostriatal cell loss is complete by Day 3 after MPTP administration and persists beyond 30 days postlesioning as determined by either unbiased stereologic counting techniques (Petzinger et al., in preparation) or section-sampling techniques (Jackson-Lewis et al., 1995).

Selection of Mice and Exercise Protocol

Figure 1A outlines the experimental design of animal groups. Before MPTP lesioning, a baseline treadmill running assessment was conducted to insure that all animals performed similarly on the treadmill task before MPTP lesioning. Forty animals that could maintain a forward position on the 2.5-m treadmill belt for 5 min at 10 m/min were assigned randomly to the four groups. A non-noxious stimulus (metal-beaded curtain) was used as a tactile incentive to prevent animals from drifting back on the treadmill. Consequently, shock-plate incentive was not used and stress related to the activity was minimized. Exercise was initiated 4 days after saline or MPTP lesioning. For exercise training, a motorized, 10-lane rodent treadmill (Fig. 1B) was used at incremental speeds to a goal treadmill speed between 20.5–23.0 m/min (Fukai et al., 2000). All 10 mice from each of the two exercise groups (MPTP + exercise and saline + exercise) were run at the same time in the 10-lane treadmill (see Fig. 1B). Exercise duration was incrementally increased to reach the goal duration of 2 × 30 min/day (60 min), 5 days/week (with a 2-min warmup period) for a total of 30 days of exercise. Treadmill speed for each group was increased when all 10 mice within each group maintained a forward position on the 2.5-m treadmill belt for 75% of the running period. When all 10 mice within each of the two running groups maintained a forward position 100% of the time, duration for that group was increased. Mice were exercised as a group of 10 for two 30-min sessions (total 1 hr) per day with a 30-min rest period between sessions. To control for any non-exercise effects of treadmill running (handling, novel environment, noise, and vibration) nonexercised groups were placed on the top of the treadmill apparatus for a time period equivalent to
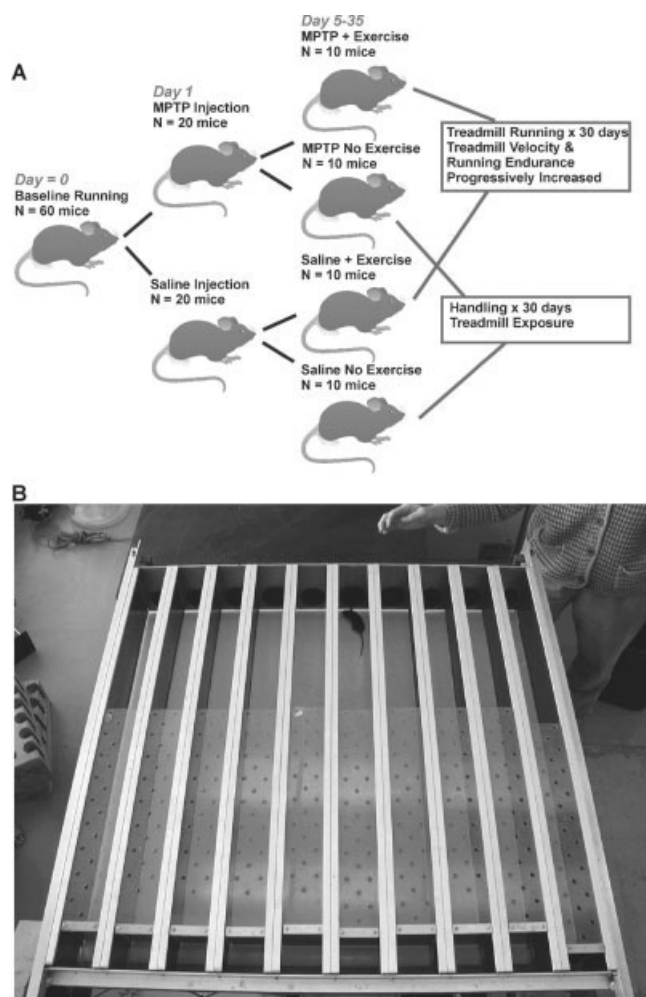
Fig. 1. **A:** Summary of experimental design with group assignment and exercise protocol. **B:** The 10-lane motorized treadmill used for exercising the mice.

exercise training (Fukai et al., 2000; Kojda et al., 2001). At the end of the 30-day running period, all animals from the four groups (exercise and non-exercise, with and without MPTP) were run to compare running speed capability. Initial treadmill velocity was set at the same speed at which initial pre-exercise baseline running capability was determined (i.e., 10 m/min). Maximum velocity for each group was defined as the velocity at which the mice, as a group of 10, could maintain a forward position on the treadmill for 75% of a 5-min running trial.

Collection of Brain Tissue

Brain tissue was collected at 4 days post-MPTP lesioning to examine the reduction in the degree of DAT and TH immunoreactivity. Brain tissue was collected from all groups at the conclusion of the 30 days of treadmill exercise, (35 days post-MPTP lesioning). Tissue for immunohistochemical analysis was fixed by transcardial perfusion with 50 ml of ice-cold saline followed by 50 ml of 4% paraformaldehyde/phosphate-

buffered saline (PFA/PBS) pH 7.2. Brains were removed, postfixed in 4% PFA/PBS for 48 hr, cryoprotected in 20% sucrose for 24 hr, and then quickly frozen in isopentane on dry ice. Tissues for Western immunoblotting and in situ hybridization were harvested fresh after cervical dislocation. All procedures used in these studies adhered to the guidelines of the Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health. To maximize the utilization of brain tissues each technique involving either fresh tissues (for Western immunoblotting), quick frozen tissues (for in situ hybridization histochemistry), or perfusion fixed (for immunoelectron microscopy or immunohistochemistry) consisted of at least 6 to as many as 10 mice from each group in a single experimental cohort. This assured a large enough *n* to detect changes within each experimental design.

Immunohistochemistry

Fixed tissue from at least six mice from each group was cut at 30-μm thickness, placed in phosphate buffer, and used immediately for immunohistochemistry. Commercially available antibodies included rabbit polyclonal anti-TH (Chemicon, Temecula, CA), and mouse monoclonal anti–DAT (Chemicon). Tissue sections were washed in Tris-buffered saline (TBS; 50 mM Tris pH 7.4 and 0.9% NaCl) and exposed to antibody (1:1,000) for 48 hr at 4°C. Sections were washed in TBS, and exposed to horseradish peroxidase (HRP)-conjugated secondary antibody using the ABC Elite kit (Vector Labs, Burlingame, CA). Antibody staining was visualized by development in DAB/H_2O_2. To ensure that differences in staining intensity were due to differences in antigen expression, multiple sections from each of the different treatment groups were handled concurrently in identical staining conditions. Control experiments excluding either primary antibody or secondary antibody were also carried out to verify staining specificity. Determination of the relative expression of TH immunoreactivity (ir) and DAT-ir in the striatum using immunohistochemistry from different mouse groups was based on published validity studies (Burke et al., 1990). For image analysis, three or four animals per treatment group and 10−12 sections per animal, spanning the midstriatum rostral to the anterior commissure (Bregma 0.25–1.25 mm) were used. Striatal images were captured at low magnification and digitized. The relative optical density (OD) (expressed as arbitrary units within the linear range of detection) of the dorsal lateral striatum was determined by subtracting the relative optical density of the corpus callosum as background. To ensure that the gray values represented an OD within the nonsaturated range of the image analysis, a Kodak Photographic step tablet (density rage to 255 OD units) captured by the CCD camera was used. Maximal tissue immunostaining relative OD units did not exceed the relative OD units of the tablet.

Western Immunoblotting

Tissue for Western blot analysis was dissected from the mid-striatum (a 3-mm thick section between Bregma 0.00−1.50) of at least six mice from each group and homogenized in buffer (25 mM Tris pH 7.4, 1 mM EDTA, 100 μM phenylmethylsulfonylfluoride [PMSF]). Protein concentration was determined by the BCA method (Pierce, Inc.). Proteins (10 μg) were

separated by polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli (1970) and transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al., 1979). Filters were blocked in TS-Blotto (50 mM Tris pH 7.4, 0.9% NaCl, 5% nonfat milk) for 1 hr, then exposed to primary antibody (1:2,000) in TS for 2 hr. Filters were then washed in TS, and exposed to secondary antibody in TS-Blotto for 1 hour. After a final wash in TS, antibody binding was visualized by chemiluminescence (Pierce, Inc.) and apposing filters to film (Hyperfilm ECL; Amersham) and processed in X-OMAT developer. Images were scanned and the relative OD of bands (expressed as arbitrary units within the linear range of film) was determined using Bioquant Nova Prime, a computer-assisted image analysis program (Bioquant Imaging, Nashville, TN).

Electron Microscopy/Immunocytochemistry

Electron microscopic immunolabeling for glutamate was carried out on mice from the saline group ($n + 6$), saline + exercise group ($n + 10$), MPTP group ($n + 7$), and MPTP + exercise group ($n + 9$). Anesthetized mice were perfused transcardially with 6 ml of heparin (1,000 U/ml) in HEPES buffer (pH 7.3) followed by 50 ml of 2.5% glutaraldehyde/0.5% paraformaldehyde in HEPES (pH 7.3) containing 0.1% picric acid. The brain was removed and post-fixed overnight at 4°C. Vibratome sections (200-mm thick) were cut in the coronal plane through the striatum and the dorsal hippocampus. A 2×2 mm^2 piece of the dorsolateral striatum (site of the major input of the corticostriatal pathway) and the CA1 subregion of the hippocampus (used as a control area to look for nonspecific effects of exercise), were dissected, washed in HEPES buffer, incubated at room temperature in the dark in aqueous 1% osmium tetroxide/1.5% potassium ferricyanide, washed in deionized water and en block stained with aqueous 0.5% uranyl acetate at room temperature for 30 min. The tissue was dehydrated, embedded in Embed 812/Spurr's (EMS; Fort Washington, PA) and sections were cut and stained. Post-embedding immunogold electron microscopy was carried out according to a modified method of Phend (Phend et al., 1992; Tillerson et al., 2003). Thin sections (light gold interface color) were cut and placed on 200-mesh nickel coated grids double coated previously with a solution from a Coat-Quick "G" pen (Kiyota International, Elk Grove, IL.), air dried for several hours, and washed for 5 min in TBS with Triton X-100 (TBST; 0.05 M Tris, pH 7.6, 0.9% NaCl, and 0.1% Triton X-100). The grids were transferred to the primary antibody solution and incubated overnight in a moist chamber. The glutamate antibody (non-affinity purified, rabbit polyclonal; Sigma, St. Louis, MO), as characterized previously by Hepler et al. (1988), was diluted 1:400,000 in TBST 7.6. Aspartate (1 mM) was added to the glutamate antibody mixture 24 hr before incubation with the thin-sectioned tissue to prevent any cross-reactivity with aspartate within the tissue. The grids were incubated for 1.5 hr at room temperature in goat anti-rabbit IgG conjugated to 10-nanometer gold (diluted 1:50 in TBST 8.2; Amersham). Photographs (10/animal) were taken randomly throughout the section containing the caudate nucleus or the CA1 region of the hippocampus (1 section/grid, one photograph per grid square) at a final magnification of 40,000× within the area of the neuropil

(location of the greatest number of synapses) by an individual blinded to the particular experimental group and then captured on the computer using an AMT (2K × 2K) digital camera (Danvers, MA). The number of gold particles per nerve terminal associated with an asymmetrical (glutamate) synaptic contact and the area of the nerve terminal was determined using Image Pro Plus imaging software (Media Cybernetics, Tacoma, WA). The gold particles contacting the synaptic vesicles within the nerve terminal were counted and considered part of the vesicular or neurotransmitter pool by previously determined methods (Meshul et al., 1998; Tillerson et al., 2003). The specificity of immunolabeling for the glutamate antibody was established by incubating the antibody overnight with 3 mM glutamate. Preabsorption of the glutamate antibody resulted in a total lack of tissue labeling. The density of glutamate immunolabeling within the mitochondria associated with the presynaptic terminal was determined also as a means of investigating changes in the presynaptic metabolic pool of glutamate. The mean density of gold particles/μm^2 $\pm$ SEM) was determined within each treatment group. Stereologic analysis was not carried out for the nerve terminal glutamate immunolabeling study, because synapse density was not being determined, only the density of gold particles per identified nerve terminal making an asymmetrical synaptic contact.

In Situ Hybridization

Brains for in situ hybridization were removed quickly and frozen in isopentane on dry ice. Sections were cut 14-μm thick on a Jung 1850 cryostat (Leica, Inc.) and thaw mounted onto poly-L-lysine-coated microscope slides, dried on a 55°C slide warmer, and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.2. Sections were dehydrated in successive ethanol washes (30, 60, 80, 95, and 100%), deprotonated in triethanolamine/acetic anhydride, delipidated in chloroform, and dehydrated in ethanol. Slides containing tissue sections were exposed to hybridization buffer containing 4× standard sodium citrate (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50% formamide, 1× Denhardt's Solution, 250 μg/ml tRNA, 500 μg/ml salmon sperm DNA, and 10% dextran sulfate with 1×10^6 cpm of probe. Sections were cover-slipped and incubated overnight at 44°C. Tissue sections labeled with ribonucleotide probes were washed first in 2× SSC/50% formamide/0.1% β-mercaptoethanol at 37°C for 30 min, then 20 μg/ml RNase in 0.5 M NaCl/10 mM Tris pH7.4 at 37°C for 30 min, followed by 2× SSC/50% formamide/0.1% β-mercaptoethanol at 60°C for 1 hr, 0.1× SSC/0.1% β-mercaptoethanol at 65°C for 1 hour, and finally rinsed in ethanol before air drying. Slides were placed against high-resolution film (Hyperfilm B-max; Amersham) with radioactive standards (Amersham, Inc.). Selected slides were dipped in NTB-2 (Kodak) photographic emulsion, developed in D-19 developer and counter stained with eosin. To minimize potential sources of variation between different experiments, slides that were to be compared were processed in the same experiment using identical hybridization cocktail, probe concentration, probe preparation, wash regimen, and film exposure. The computerized image analysis program Bioquant was used to

determine the number of emulsion grains above specific anatomic regions within the substantia nigra pars compacta.

Statistical Analysis

Linear regression was carried out to compare the rate of change in velocity and endurance of treadmill running between the two groups. Treatment groups were compared using one-way analysis of variance (ANOVA), followed by the Fisher post hoc test for comparison of multiple means for the following measures: DAT, TH, dopamine D1 and D2 receptors, and glutamate immunogold labeling. All analyses were carried out with SPSS software. Statistical significance was accepted at $P < 0.05$.

RESULTS

Exercise-Induced Changes in Behavior

Figure 1 outlines the four animal groups used in this study. Two groups (saline + exercise and MPTP + exercise) were subjected to treadmill exercise for 30 days and changes in running duration and velocity were measured. Duration increased over the 30-day exercise period for both groups (see Fig. 2A). Both the saline + exercise and MPTP + exercise groups were capable initially of running for a duration of 30 min on Day 1, which increased to a maximal duration of 60 min. The saline + exercise group reached maximal duration by Day 12, however, whereas the MPTP + exercise group did not reach maximal duration until Day 26. As such, we compared the rate of increase in exercise duration over the first 12 days of running. The rate of change of duration for the saline + exercise group was significantly greater than that seen in the MPTP + exercise group ($P < 0.05$).

Similar to duration, running velocity increased in both the saline + exercise and MPTP + exercise groups over the 30-day period (see Fig. 2B). The saline group ran at a velocity of 13.3 m/min at Day 1 and increased to 23.0 m/min by Day 30. The MPTP group ran at a velocity of 6.3 m/min at day 1 and increased to 21.7 m/min by day 30. There was a significant difference in velocity at Day 1 between the two groups (saline + exercise, 13.3 m/min; MPTP + exercise
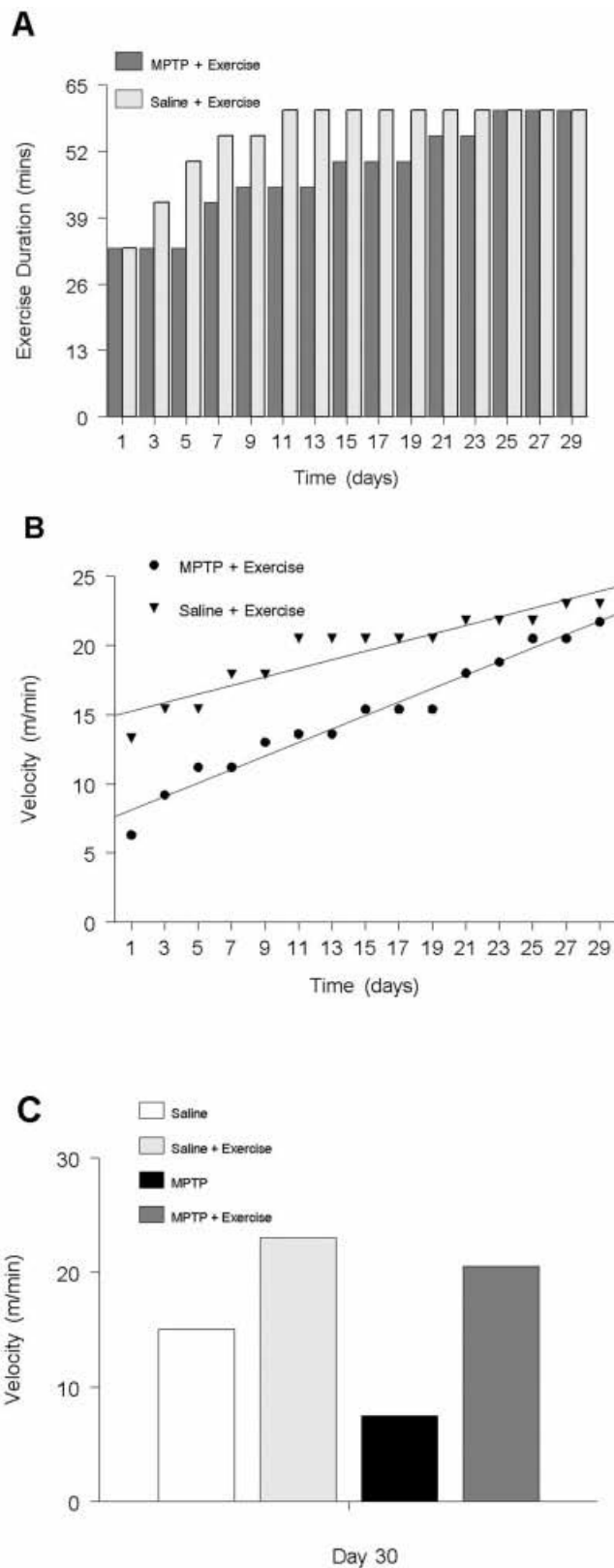

Fig. 2. Exercise-induced changes in behavior. **A:** Change in running duration over the 30-day running period for the saline + exercise group (gray bars) and MPTP + exercise group (black bars). The bars represent the performance of all 10 mice/group running at the same time. The increase over days of running reflects that all 10 mice met the criteria for increasing running duration. No statistical analysis was carried out because each bar represents all 10 mice/group as a single data point. **B:** Change in running velocity (in m/min) over the 30-day running period for the saline + exercise group (triangles) and MPTP + exercise group (circles). Symbols represent the performance of all 10 mice in each running group; increase over days of running reflects that all 10 mice met the criteria for increasing running velocity. **C:** Compares running velocity between the four groups (saline, white bar; MPTP, light gray bar; saline + exercise, black bar; and MPTP + exercise, dark gray bar) at the conclusion of the running program on Day 30. The bars represent performance of all 10 mice/group from the four groups running at the same time.
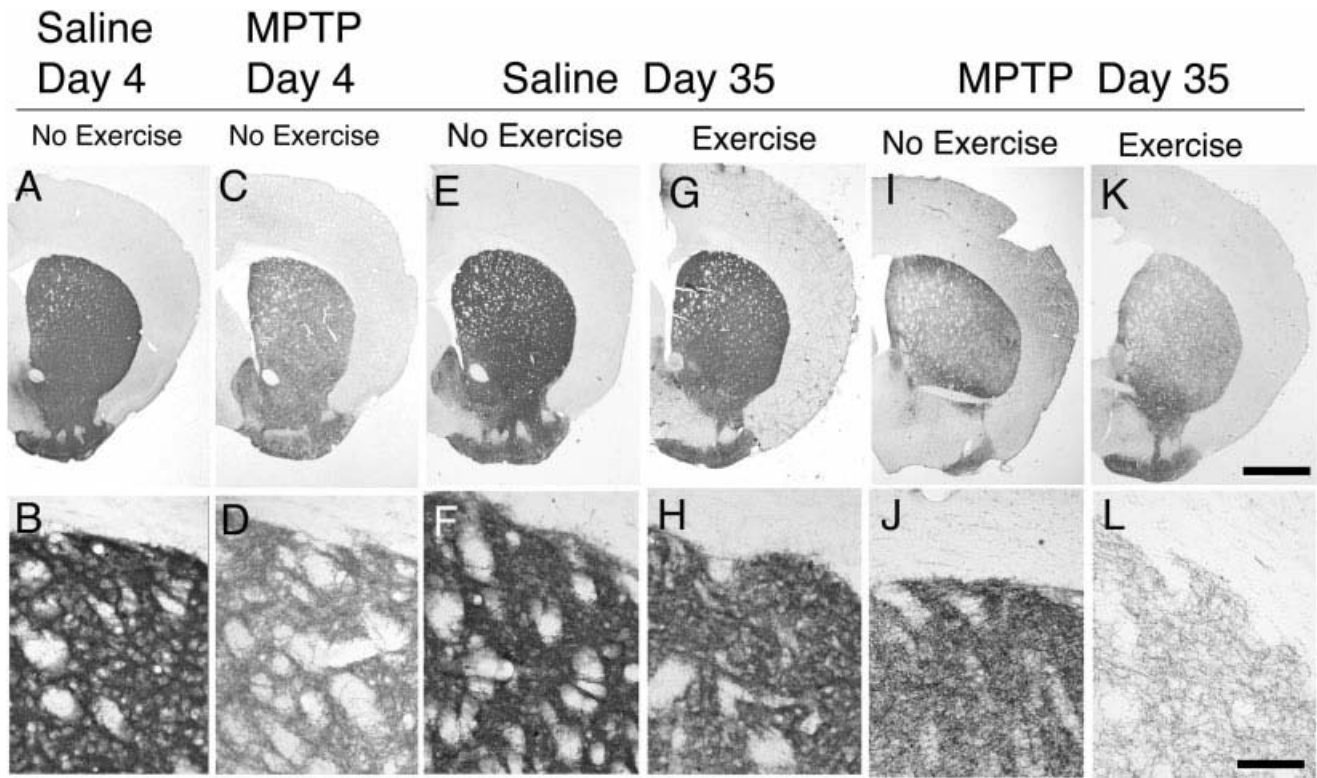
Fig. 3. Exercise-induced changes in striatal dopamine transporter immunolabeling. The relative expression of striatal dopamine transporter protein (DAT) after MPTP lesioning and between different treatment groups was determined using immunohistochemistry. The upper panels show low-magnification (10×) images of coronal sections at the level of the midstriatum stained with an antibody against DAT protein. The lower panels show high-magnification (400×) images from a region corresponding to the dorsal lateral striatum from respective sections in the upper panels. MPTP lesioning leads to a reduction in DAT-ir (comparing saline in **A** and **B** and MPTP in **C** and **D** at Day 4). MPTP + exercise showed reduced DAT-ir (**K** and **L**) compared to MPTP without exercise (**I** and **J**). Saline + exercise (**G** and **H**) showed a slight reduction in DAT-ir compared to saline alone (**E** and **F**). Scale bar + 0.5 mm (in K, for upper panels); 50 μm (in H, for lower panels).

group, 6.3 m/min; 7.0 m/min difference between groups at Day 1; $P < 0.0001$). This difference reduced to 1.3 m/min by Day 30 (Fig. 2B). The change in velocity over days of running resulted in a significantly different rate of change between the two groups ($P < 0.0001$). The MPTP + exercise group increased treadmill velocity by 5 m/min per day compared to 3.1 m/min per day for the saline + exercise group (Fig. 2B).

At the end of the 30-day running period, all animals from the four groups (saline, saline + exercise, MPTP, and MPTP + exercise) were tested on the treadmill to compare running speed capability (Fig. 2C). The MPTP and MPTP + exercise groups had a treadmill velocity of 7.5 and 21.7 m/min, respectively. Interestingly, the running velocity of the MPTP group at 35 days post-MPTP lesioning (7.5 m/min) was similar to the MPTP + exercise group at Day 1 (6.3 m/min) of their treadmill exercise program. Taken together, these findings indicate that there was no spontaneous increase in running velocity in the MPTP nonexercised group. The intensity of our training regimen was substantiated by the fact that there was an effect of exercise in the saline + exercise group compared to the saline nonexercised group. The comparison of running speed capability (Fig. 2C) at the end of the exercise program demonstrated differences in running velocity between the saline groups (saline + exercise, 23 m/min; saline nonexercised, 15 m/min).

Exercise-Induced Changes in Striatal Dopamine Transporter Protein

Analysis of the pattern of expression of DAT protein in the midstriatum (Bregma level +1.00) in both the saline (Fig. 3A,B) and MPTP groups (Fig. 3C,D) at Day 4 showed a significant reduction in DAT-ir due to MPTP lesioning. After the exercise regimen (Day 35 post-lesioning) the saline group showed the highest degree of striatal DAT-immunoreactivity (DAT-ir) compared to that in all other groups (Fig. 3E,F). Interestingly, the saline + exercise group had reduced DAT-ir compared to the saline group, which suggests that exercise itself reduced DAT-ir (compare Fig. 3E,F with Fig. 3G,H). This reduction was shown to be significant (saline, $100.0 \pm 3.1\%$; saline + exercise, $84.3 \pm 2.8\%$; $P < 0.006$) (see Fig. 4). In addition, the MPTP + exercise group demonstrated sig-
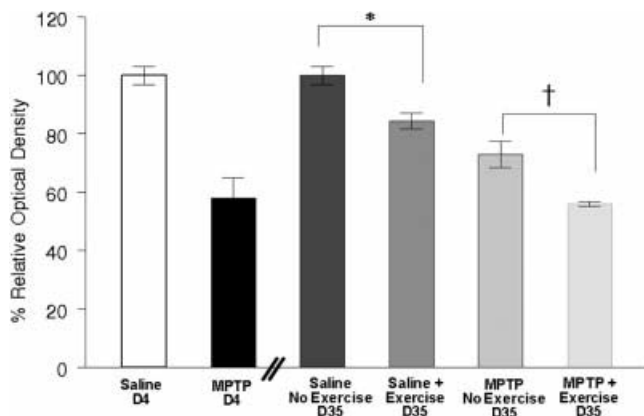
Fig. 4. Analysis of exercise-induced expression of dopamine transporter immunolabeling: The relative striatal DAT-ir was determined by measuring the relative optical density of the dorsal lateral quadrant (in at least 12 sections) correcting for staining background within sections by measuring the relative OD of the corpus callosum. This data represents relative staining for representative sections from the saline and MPTP groups at Day 4 post-MPTP lesioning and the four treatment groups collected after completion of the exercise program at post-MPTP lesioning Day 35. For comparisons, the Day 4 saline group was arbitrarily set as 100% (mean ± SEM, 100 ± 3.1%) and the relative optical densities of all other groups were normalized against it. Comparison of DAT-ir at Day 4 post-MPTP lesioning (58.0 ± 7.0%) showed a significant reduction in DAT-ir. The asterisks and cross indicate statistically significant differences between the saline and saline + exercise and the MPTP and MPTP + exercise groups, respectively. This reduction was significant (saline, 100.0 ± 3.1%; saline + exercise, 84.3 ± 2.8%; $P < 0.006$). In addition, the MPTP + exercise group demonstrated significantly reduced DAT-ir compared to the MPTP group (MPTP, 73.3 ± 4.5%; MPTP + exercise, 56.0 ± 0.7%; $P < 0.028$).
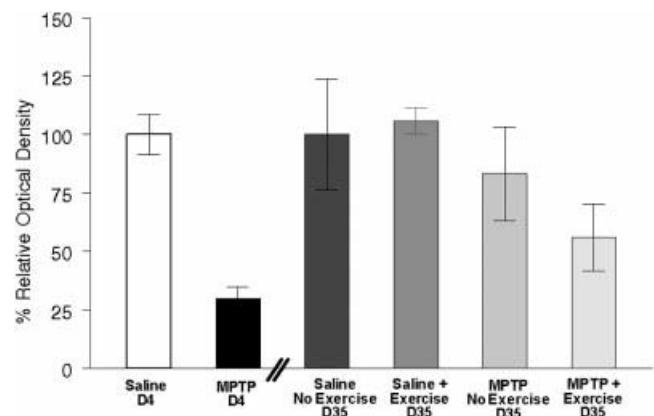

Fig. 5. Analysis of the relative striatal TH-protein expression by Western immunoblotting. The relative optical density measurement of the saline group at Day 4 post-lesioning was set arbitrarily at 100 ± 3.1% for comparison between different treatment groups. Analysis of TH-protein levels at 4 days after MPTP lesioning showed a significant reduction (30.0 ± 4.5%) compared to that in saline controls. There were no significant differences between groups in TH-ir at 35 days post-MPTP lesioning (saline, 100.0 ± 23.8%; saline + exercise, 105.8 ± 5.7%; MPTP, 83.3 ± 20.0%; MPTP + exercise, 56.0 ± 14.3%; $P + 3.72$). These data, however, indicate a small, nonsignificant reduction in the relative density of TH-ir in the MPTP groups compared to that in the saline groups. In addition, TH-ir was reduced slightly in the MPTP + exercise group compared to MPTP alone.

nificantly reduced DAT-ir compared to the MPTP group (MPTP, 73.3 ± 4.5%; MPTP + exercise, 56.0 ± 6.3%; $P < 0.028$) (see Fig. 4 and compare Fig. 3I,J with Fig. 3K,L).

Exercise-Induced Changes in Tyrosine Hydroxylase Striatal Protein

Western immunoblotting with an antibody recognizing TH protein was carried out on striatal tissue at post-lesioning Day 4 and at the end of the exercise program (35 days after MPTP lesioning) (Fig. 5). Comparison of the Day 4 saline with the Day 4 MPTP lesioning showed a significant reduction in striatal TH protein due to MPTP lesioning (saline, 100 ± 3.0% compared to MPTP, 30.0 ± 4.5%). Comparison of the level of TH protein at the end of the running regimen (at Day 35) showed that there was no significant difference in TH immunoreactivity between any of the groups (saline, 100.0 ± 23.8%; saline + exercise, 105.8 ± 5.7%; MPTP, 83.3 ± 20.0%; MPTP + exercise, 56.0 ± 14.3%, $P + 3.72$). Although not significant (35 days after MPTP lesioning), MPTP groups (MPTP and MPTP + exercise)

showed a slight reduction in TH immunoreactivity compared to the saline groups. In addition, TH immunoreactivity was reduced slightly in the MPTP + exercise group compared to that in the MPTP group.

Exercise-Induced Changes in Dopamine D1 and D2 Receptors

In situ hybridization histochemistry with probes recognizing either the D1 or D2 subtype of dopamine receptor was carried out on dorsal striatal tissue of mice from all groups. Compared to the saline group, expression of D1 mRNA was reduced as a result of exercise, MPTP lesioning, or both (saline, 100.0 ± 6.9%; saline + exercise, 51.9 ± 3.9%; MPTP, 50.1 ± 5.9%; MPTP + exercise, 48.6 ± 4.2%; $P < 0.0001$) (Fig. 6A). Comparison of the MPTP with the MPTP + exercise group showed no difference in the level of dopamine D1 mRNA expression. The expression of dopamine D2 mRNA was also reduced as a result of either exercise (saline + exercise) or MPTP lesioning (MPTP group) compared to that in the saline group (saline, 100.0 ± 7.6%; saline + exercise, 58.5 ± 6.4%; MPTP, 50.1 ± 5.7%; $P < 0.002$) (Fig. 6B). The combination of MPTP lesioning and exercise (MPTP + exercise group), however, resulted in no difference in dopamine D2 mRNA expression when compared to that in the saline group, but was increased significantly when compared to that in the MPTP group (MPTP + exercise, 95.9 ± 9.4%; $P < 0.005$).
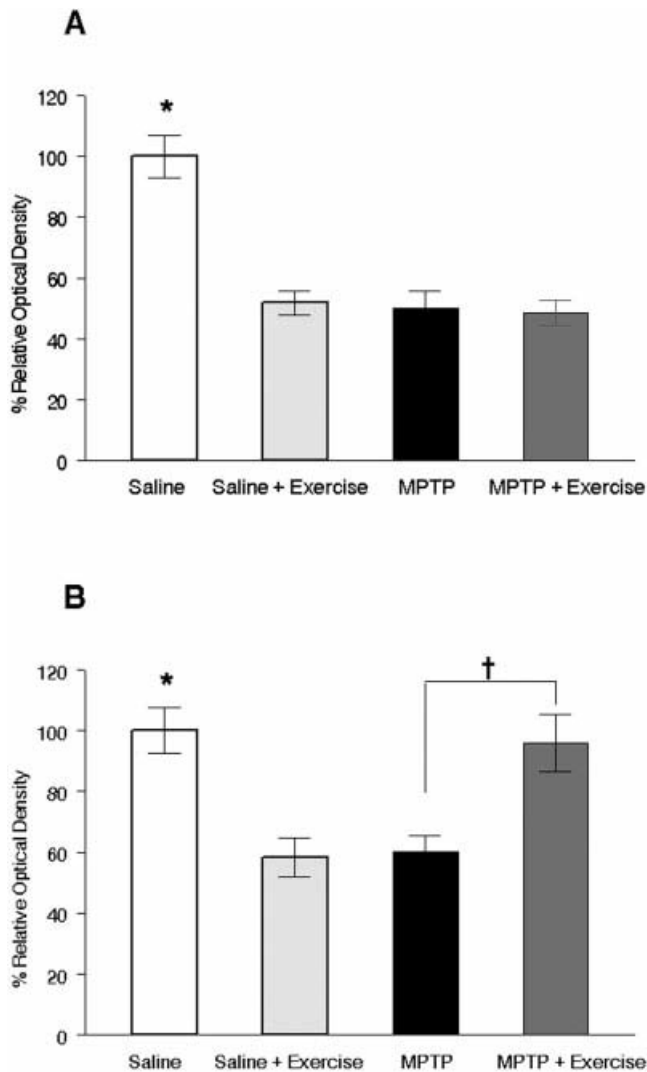
Fig. 6. Analysis of the relative striatal dopamine D1 and D2 receptor mRNA using in situ hybridization histochemistry. The relative optical density of autoradiographic grains above the dorsal striatum were determined from at least three mice from each treatment group using at least 12 sections/mouse. For comparison, the saline group was set arbitrarily at 100% and all other groups normalized against it for both dopamine receptors D1 and D2 mRNA. **A:** Compared to the saline group, expression of D1 mRNA was reduced significantly with exercise and MPTP lesioning (saline, $100.0 \pm 6.9\%$; saline + exercise, $51.9 \pm 3.9\%$; MPTP, $50.1 \pm 5.7\%$; MPTP + exercise, $48.6 \pm 4.2\%$; $P + 0.0001$). **B:** The expression of dopamine D2 mRNA was reduced as a result of either exercise (saline + exercise) or MPTP lesioning (MPTP group) compared to that in saline group (saline, $100.0 \pm 7.6\%$; saline + exercise, $58.5 \pm 6.4\%$; MPTP, $60.3 \pm 5.3\%$; $P < 0.002$). The expression of dopamine D2 mRNA was increased significantly in the MPTP + exercise group when compared to that in the MPTP group (MPTP + exercise, $95.9 \pm 9.4\%$; $P < 0.005$).

Exercise-Induced Changes in Nerve Terminal Glutamate Immunolabeling

Immunogold electron microscopy was used to determine the density of nerve terminal glutamate immuno-

labeling in mice from all groups at completion of the exercise program. Figure 7 shows representative images of asymmetrical (excitatory) synaptic contacts labeled for the neurotransmitter glutamate in mice from the saline group, saline + exercise group, MPTP group, and the MPTP + exercise group (Fig. 7A–D, respectively). There was a significant increase in the density of nerve terminal glutamate immunolabeling in the MPTP group compared to saline, (values are mean number of gold particles/$\mu m^2 \pm$ SEM: saline, 85.9 ± 3.6; MPTP, 135.3 ± 12.4; $P < 0.05$) (Fig. 8). Additionally, there was a significant decrease in the density of nerve terminal glutamate immunolabeling in the MPTP + exercise group compared to the MPTP group (MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5; $P < 0.05$). This decrease reached levels that were similar to the saline groups (mean number of gold particles/$\mu m^2 \pm$ SEM: saline, 85.9 ± 3.6; saline + exercise, 102.6 ± 3.7; MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5).

To determine the specificity of the change in the density of glutamate immunolabeling within the nerve terminal, the density of labeling within the presynaptic mitochondrial pool was quantified. There was no difference between any of the experimental groups (data not shown). Additionally, there were no changes in nerve terminal area between groups (data not shown).

Glutamate immunolabeling of the CA1 in the hippocampus was analyzed to confirm that the observed differences between groups in glutamate immunolabeling were specific to the striatum. This brain area was chosen because it also receives a significant glutamatergic input and is associated with spatial learning as opposed to the dorsolateral striatum that is associated primarily with motor function. There were no differences in the density of nerve terminal glutamate immunolabeling between any of the groups as shown in Figure 8B (values are mean number of gold particles/$\mu m^2 \pm$ SEM; saline, 100.0 ± 5.7; saline + exercise, 101.7 ± 7.9; MPTP, 88.4 ± 4.6; MPTP + exercise, 103.2 ± 6.6; $P < 0.49$).

DISCUSSION

The purpose of this study was to examine the effect of exercise on restoration of surviving dopaminergic neurons after completion of MPTP-induced cell death. The MPTP lesioning regimen used in our studies involves a series of four injections of 20 mg/kg (free-base) leading to a 60–70% loss of nigrostriatal dopaminergic neurons and a 90–95% depletion of striatal dopamine (Jackson-Lewis et al., 1995). Using this regimen, nigrostriatal cell loss is complete by Day 3 after MPTP administration and shows no further decline 30 days post-lesioning as determined by either unbiased stereologic counting techniques (Petzinger et al., in preparation) or section-sampling techniques (Jackson-Lewis et al., 1995). Despite the extent of cell loss, MPTP-lesioned mice display robust and reproducible return of striatal function 2–3 months after injury (Ricaurte et al., 1986; Jakowec et al., 2003). The levels of TH and DAT immunoreactivity (TH-ir and DAT-ir) are de-
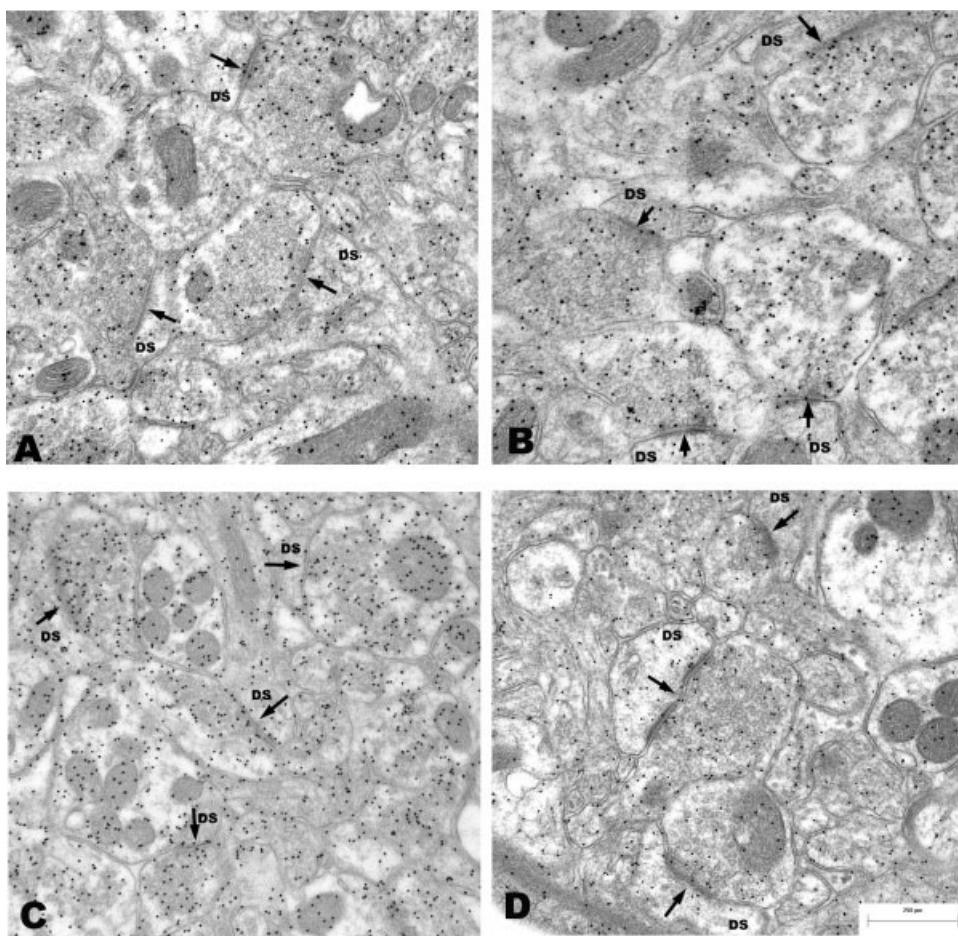
Fig. 7. Electron photomicrographs using the immunogold technique to localize an antibody against the neurotransmitter, glutamate, within the dorsolateral striatum. **A:** Saline group. Three nerve terminals are seen making an asymmetrical synaptic contact (arrows) with an underlying dendritic spine (DS). Within the nerve terminal are numerous 10-nm gold particles, indicating the location of the antibody. These gold particles are found overlying the round synaptic vesicles. **B:** Saline group that was exercised for 30 days, starting 4 days after the injection of saline. Note that the density of nerve terminal glutamate immunolabeling seems similar to that seen in the saline-treated group in A. **C:** MPTP-treated group was given an acute injection of the toxin (20 mg/kg $\times$ 4 injections every 2 hr) and then perfused with fixative 34 days later. Note the increase in the density of immunogold particles in all three nerve terminals compared to that observed in the saline group shown in A. **D:** MPTP-treated group that was exercised for 30 days, starting 4 days after the acute toxin treatment. Note that the density of glutamate immunogold labeling is similar to that seen in the saline-treated group in A. Scale bar + 0.25 μm.

creased to 30% of pre–MPTP-lesioned levels by Day 7 and return to 50–60% or more of pre–MPTP-lesioned levels within 30–60 days (Jakowec et al., 2003, 2004). These changes are part of molecular alterations underlying intrinsic neuroplasticity in this model (Jakowec et al., 2004) and are represented in this study by the MPTP nonexercised group. The saline group in this study served two purposes: (1) to show that the intensity of the exercise regimen was sufficient to induce a behavioral effect in non-lesioned controls; and (2) to compare the effect of exercise on the noninjured and injured brain.

Using a high-intensity (high velocity and duration) treadmill exercise paradigm in the MPTP-lesioned mouse, we have shown that exercise leads to behavioral recovery, specifically amelioration of initial deficits in running speed and duration compared to nonexercised MPTP-lesioned animals. Importantly, as would be expected by an effective training paradigm, the non-lesioned animals that exercised demonstrated enhanced performance compared to their non-lesioned, nonexercised counterparts. Specifically these behavioral differences between exercised and non-exercised mice consisted of the capability of the exercised saline mice to run at higher velocities after a 30-day treadmill-training program. We showed that MPTP-lesioned and non-lesioned mice could be forced to run at progressively faster speeds and longer durations and learn to associate a sensory stimulus with a behavioral response (i.e., maintaining a specific position on the treadmill). Over time, sensory feedback was no longer necessary for the animals to maintain a forward position, indicating that learning had occurred.

Although the MPTP-lesioned mouse displays subtle motor behavioral deficits that may not be evident under normal caging conditions, motor deficits become evident under specific task or environmental manipulations (Sedelis et al., 2001; Tillerson et al., 2002). Bradykinesia (slowness of movement) and fatigability (decreased endurance) were two behavioral deficits we observed initially in treadmill-exercised mice after MPTP lesioning. MPTP-lesioned mice were not capable of running at the same treadmill velocity as the saline + exercise mice in the first 18 days of running. By the end of the 30-day exercise program, the MPTP + exercise mice were running at a velocity near that of the saline + exercise group and greater than that of the saline (nonexercised) group (see Fig. 2C). The rate of change of velocity was greater in the
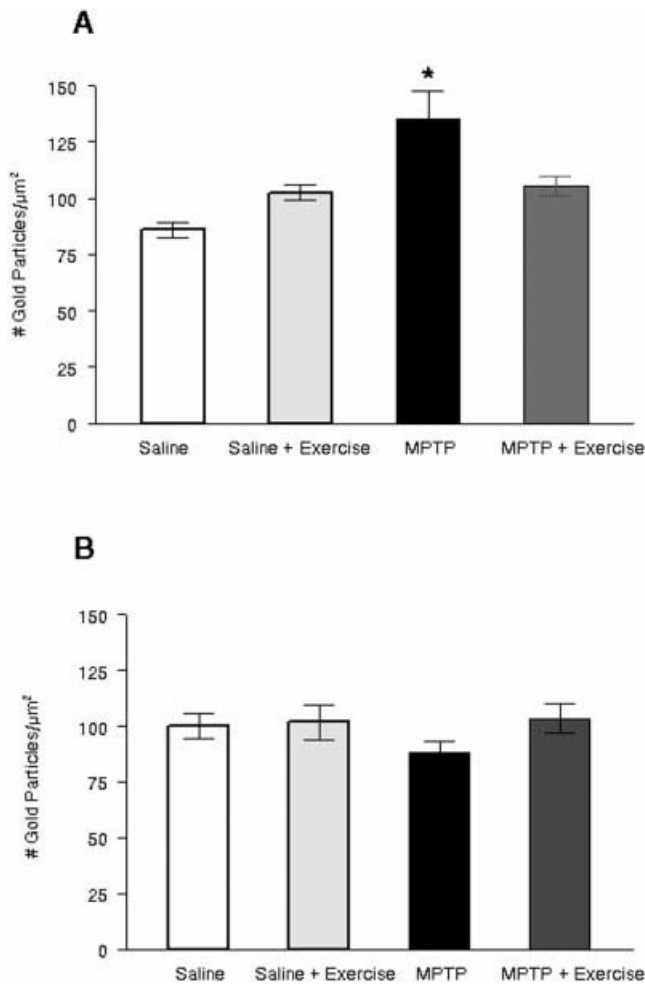
Fig. 8. Quantification of immunogold electron microscopy results. **A:** There was a significant increase in the density of synaptic glutamate immunolabeling within striatal nerve terminals making asymmetrical synaptic contacts after the acute administration of MPTP compared to the other treatment groups (mean number of gold particles/μm^2 ± SEM: saline, 85.9 ± 3.6; saline + exercise, 102.6 ± 3.7; MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5; $P < 0.05$). **B:** Using quantitative immunogold electron microscopy, there was no difference in the density of glutamate immunolabeling within nerve terminals making an asymmetrical synaptic contact within the CA1 region of the hippocampus between any of the treatment groups. ★$P < 0.05$ compared to all the other groups as determined by the Fisher post-hoc test for comparison of multiple means.

injured animals compared to their nonlesioned exercised counterparts. This result is in accordance with exercise studies in cortically injured animals in which the effect of exercise is greater after injury. Injury may prime the system for adaptation perhaps through the induction of neurotrophic factors including brain-derived neurotrophic factor (BDNF) (Cotman and Berchtold, 2002; Gomez-Pinilla et al., 2002; Cohen et al., 2003).

Tillerson et al. (2003) also reported behavioral improvement after treadmill exercise in two rodent models

of basal ganglia injury (the 6-OHDA rat and MPTP-lesioned mouse), which was associated with attenuation of dopamine loss (Tillerson et al., 2003). The investigators concluded that exercise might work largely through neuroprotective mechanisms because exercise was started within 12 hr of lesioning, and MPTP and 6-OHDA may take several days to complete cell death (Sauer and Oertel, 1994; Jackson-Lewis et al., 1995). Unlike the Tillerson et al. (2003) study, the focus of our study was to address the effect of high-intensity treadmill exercise on the neuro-restoration of surviving neurons after MPTP lesioning. This was accomplished by: (1) initiating exercise 4 days after lesioning, a time period well after cell death is completed in this model; (2) continuing exercise over a 30-day period; and (3) progressively increasing treadmill velocity and duration over that period. Two additional differences between Tillerson et al. (2003) and the present study were the exercise parameters and the age of the animals. Our exercise parameters on young mice were of higher velocity, duration, and frequency and showed an effect on the saline + exercise group that was not seen in the Tillerson et al. (2003) study.

In addition to a behavioral effect, exercise resulted in decreased DAT-ir compared to the nonexercised groups. The effect of exercise on DAT-ir was even greater in the MPTP group. In the basal ganglia, the biosynthesis of dopamine is dependent on the enzyme tyrosine hydroxylase (TH) and the primary mechanism of clearance of dopamine from the extracellular space is through the dopamine transporter (DAT) (Gainetdinov et al., 2002; Mortensen and Amara, 2003). Several mechanisms have been shown to regulate DAT activity including: (1) gene and protein expression of transporter number; (2) phosphorylation activated through glutamate receptors such as the mGluR5 metabotropic receptor; and (3) internalization within endosomes mediated by dopamine (Perrone-Calano et al., 1996; Page et al., 2001). Alterations in DAT activity can influence the synaptic occupancy of dopamine. An intervention (such as exercise) that downregulates DAT-ir expression may therefore lead to behavioral improvement by increasing synaptic occupancy of dopamine. The downregulation of DAT-ir in our exercised animals could account for the superior running capabilities of both the MPTP and saline groups compared to the nonexercised groups. This interpretation is consistent with the findings of Meeusen et al. (1997) and others that report increased extracellular levels of dopamine with exercise. An alternative explanation for reduced DAT-ir in our exercised animals is the loss of nigrostriatal terminals where DAT normally resides. This does not seem likely because TH-ir, another marker of nigrostriatal terminal integrity, was not altered significantly and because exercised animals had superior running capability compared to their nonexercised counterparts, which would not be expected if cell death were ongoing. Studies are underway currently to investigate the possibility of cell death and terminal loss

including stereologic cell counting of substantia nigra pars compacta neurons and fiber density, respectively.

In saline animals, exercise suppressed dopamine D1 and D2 receptor mRNA levels. In the MPTP group, exercise seemed to have no effect on D1 but increased D2 mRNA levels. Of the dopamine receptor superfamily, D1 and D2 subtypes are the most prevalent in the striatum (REF). Activation of these receptors by dopamine leads to the release of neuropeptides from medium spiny neurons. Medium spiny neurons with D1 receptors express the neuropeptide preprotachykinin (PPT) and medium spiny neurons with D2 receptors express the neuropeptide preproenkephalin (PPE) (Gerfen, 2000). In the normal brain, D1 and D2 act synergistically and activation of both is required to elicit a motor response (Gerfen et al., 1995). In the lesioned basal ganglia, this synergy is lost and activation of either D1 or D2 may elicit a motor response. In addition, D2 activation alone in the injured state seems to elicit a more robust motor response that may be attributed to its heightened sensitivity after lesioning (LaHoste and Marshall, 1993). In our study, exercise seems to have a similar effect on both receptor subtypes in the saline animals that may reflect the synergy normally seen in the uninjured basal ganglia. This synergy, however, is lost in injury and affects each receptor subtype differently. The loss of synergy between the dopamine D1 and D2 receptors due to injury by MPTP is revealed in the context of exercise. One possible mechanism to explain the differential effect of exercise and injury on the dopamine receptors subtypes D1 and D2 may be through the action of glutamate. Glutamate has been shown to influence subtype-specific regulation of the dopamine receptors (see discussion below). The combination of the upregulation of D2 mRNA (leading to increased motor activity) along with the downregulation in DAT (leading to increased synaptic occupancy of dopamine) may explain the behavioral improvement seen in the MPTP + exercise mice. Studies are underway currently to localize the dopamine D2 receptor changes with exercise and to determine if D1 or D2 receptor antagonists or agonists affect the behavioral benefits of exercise.

Glutamate is the major excitatory neurotransmitter in the brain and plays an important role in motor behavior (Starr, 1995). Glutamate is stored within nerve terminals and upon release binds to a superfamily of receptors including the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), and kainic acid (KA) subtypes. In the striatum, the primary glutamatergic pathway is the corticostriatal input to the medium spiny neurons (Starr, 1995). One method for examining changes in glutamatergic neurotransmission is to measure alterations in glutamate storage within striatal nerve terminals using immunogold electron microscopy. We observed an increase in the density of nerve terminal glutamate immunolabeling in animals after MPTP lesioning. This increase was reversed by exercise to levels seen in the saline control groups. Additionally, this effect was specific to the lesioned dorsal-lateral striatum (an area associated primarily with motor function) because there was no alteration in CA1 glutamate terminals originating from either the Schaffer collaterals or from the contralateral hippocampus (an area associated primarily with learning and memory). In comparison to the MPTP-lesioned animals, no significant change in immunogold labeling was observed between the saline and saline + exercise groups. A change in glutamate terminal storage in the nonlesioned brain may require a higher intensity of exercise then used in the present studies (Meeusen et al., 1997).

Although glutamate levels were not measured in this study, Meshul et al. (2000) has shown an inverse relationship between terminal glutamate immunogold labeling and levels of glutamate within the synapse. An increase in the density of nerve terminal glutamate immunolabeling (as is seen with MPTP lesioning) may therefore reflect a decrease in the extracellular levels of striatal glutamate. Consequently, one hypothesis with respect to our results is that an effect of exercise in the MPTP-lesioned brain may be to increase the release of glutamate at the synapse, which that may alter dopamine receptor subtype expression or medium spiny neuron peptide expression (Cepeda et al., 1993; Cepeda and Levine, 1998; Liste et al., 1999). Using microdialysis in the 6-OHDA rat, Meeusen et al. (1997) showed an increase in extracellular glutamate with exercise (Meeusen et al., 1997; Bland et al., 1999).

Studies have shown that there are close interactions between glutamate and dopamine neurotransmission in mediating motor control (Starr and Starr, 1994; Starr, 1995; Starr et al., 1997). The striatal medium spiny neuron is thought to be the site for integrating these interactions. Exercise may either directly affect the medium spiny neuron or indirectly influence its afferents. For example, expression of the immediate early gene cFos (a marker of cell activation) and the neuropeptides preprotachykinin and preproenkephalin in medium spiny neurons are altered by exercise (Cepeda et al., 1993; Liste et al., 1999). Altered expression of these markers in medium spiny neuron activity can be blocked by either glutamate or dopamine receptor antagonists or through denervation. The present study has shown changes in both glutamate and dopamine systems. We do not yet know, however, if glutamate and dopamine changes are dependent or mutually exclusive of each other. To test the degree of dopamine–glutamate interactions with exercise, we are conducting additional studies with exercise in MPTP and saline mice administered either glutamate or dopamine antagonists. If the suppression of DAT-ir seen in our studies can be blocked by administration of a glutamate antagonist during exercise, it would support the hypothesis that glutamatergic neurotransmission is important in regulating exercise-induced changes in dopamine function. Furthermore, dopamine receptor-specific agonists and antagonists targeting either D1 or D2 will test whether the alterations in glutamate immunolabeling seen in our studies are dependent on dopamine neurotransmission.

In conclusion, exercise may be both neuroprotective and neurorestorative in the injured basal ganglia. It has been shown previously that initiating exercise at or during the time of neurotoxin–induced cell death is neuroprotective by attenuating striatal dopamine loss (Cohen et al., 2003; Tillerson et al., 2003). Our studies show that a high-intensity treadmill exercise paradigm initiated after the period of neurotoxin–induced cell death is neurorestorative as demonstrated through its beneficial effect on motor behavior. Alterations in both dopaminergic and glutamatergic neurotransmission in response to exercise may underlie the molecular mechanisms of this effect. The potential impact of this study is that exercise may not only play a role in prevention of Parkinson's disease but in restoring function in individuals who have been diagnosed with Parkinson's disease.

ACKNOWLEDGMENTS

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